COURTESY COPY OF THE PCT APPLICATION AS ORIGINALLY FILED WITH ABSTRACT

TITLE: Metabolically engineered microbial cell comprising a modified redox activity.

FIELD OF THE INVENTION

The invention is in the area of microbial biotechnology and relates to a metabolically engineered microbial cell comprising an expressible enzyme activity which, when expressed, is capable of providing an alteration in the redox level of said cell.

An alteration of said redox level comprises any increase or decrease in e.g. the rate of synthesis and/or amount produced of one or more components of a redox system such as e.g. a nicotinamide adenine dinucleotide coenzyme in its oxidised form (NAD) or reduced form (NADH), or a nicotinamide adenine dinucleotide phosphate coenzyme in its oxidised form (NADP) or reduced form (NADPH).

The increase or decrease of a rate of synthesis and/or an amount of coenzyme produced results in a redirection of a metabolic flux in said cell. The redirected metabolic flux is exploited in the generation of an increased or decreased production of a cellular metabolite.

BACKGROUND OF THE INVENTION

A complex metabolic network of more than a thousand different and simultaneously occurring reactions in a cell is regulated strictly and sensitively in order to avoid an undesirable accumulation or deficiency of metabolic intermediates and/or metabolic end products produced by said cell. A strict and sensitive regulation ensures that reactions of great mechanistic complexity and stereochemical selectivity may proceed smoothly under normal physiological conditions.

The complex metabolic network comprises numerous interrelated metabolic pathways, the totality of which is resulting in an overall cellular flux of metabolites. Any alteration of said metabolic flux may in principle be perceived as a form of metabolic engineering. Accordingly, metabolic engineering achieved e.g. by means of in vitro and/or in vivo

genetic engineering techniques represents a purposeful designing of metabolic networks and generates a change in and/or a redirection of the flow of metabolites in a microbial cell under e.g. aerobic or anaerobic conditions. Product formation of e.g. anaerobically growing microbial cells is well established in the art and has been reported in the academic literature (Oura, 1977; van Dijken & Scheffers, 1986; Nissen *et al*, 1997).

State of the art metabolic engineering strategies have been described by among others Cameron and Chaplen (1997) in Curr. Opin. Biotechnol., vol. 8, pages 175 – 180, Hahn-Hägerdal et al. (1996) in Ann. New York Acad. Sci., vol. 782, pages 286 – 296, Stephanopoulos (1994) in Curr. Opin. Biotechnol., vol. 5, pages 196 – 200, Stephanopoulos and Sinskey (1993) in Trends Biotechnol., vol. 11, pages 392 – 396, and Cameron and Tong (1993) in Appl. Biochem. Biotechnol., vol. 38, pages 105 – 140.

Several examples of metabolically engineered microorganisms are described in the prior art. EP 0 733 712 A1 discloses a method of production of preferably an amino acid by culturing a metabolically engineered microbial cell, preferably an *Escherichia coli* cell, with a supposedly increased expression or productivity of NADPH and isolating said amino acid.

WO 96/41888 discloses yeast having a modified alcohol sugar fermentation due to an altered expression of a gene encoding an NADH dependent glycerol-3-phosphate dehydrogenase activity.

EP 0 785 275 A2 discloses a yeast comprising constitutive expression of a gene encoding an enzyme activity involved in hexose transport.

EP 0 645 094 A1 discloses the use of a yeast comprising a glycolytic pathway comprising a futile cycle generated by means of a constitutive expression of genes encoding fructose-1,6-biphosphatase and phosphoenolpyruvate carboxykinase.

US 5,545,556 discloses a yeast strain having a reduced or increased production of glycerol mediated by mutations in various gene-encoded products.

Although detailed studies of microbial metabolism continue to generate an improved understanding of cellular metabolism and an increased knowledge of the complex array of chemical reactions occurring in a microbial cell, many such reactions, and in particular their regulation, are far from being thoroughly understood.

Many microorganisms are capable of growing in both the presence and absence of oxygen. However, some microbial cells are strictly aerobic and depend absolutely upon an oxidative form of metabolism known as respiration, i.e. the coupling of energy generation to an oxidation of nutrients by oxygen. During aerobic metabolism in eukaryotic microorganisms, NADH is reoxidised through the mitochondrial electron transport chain in a process that generates additional energy and results in an ultimate transfer of electrons to oxygen.

In contrast, many microorganisms either can or must grow in anaerobic environments while deriving their metabolic energy from processes that do not involve oxygen. Most of such anaerobically growing microbial organisms derive their energy from fermentations characterised by energy-yielding catabolic pathways such as glycolysis. Pyruvate formed in this pathway may be further reduced to a variety of metabolic end products such as e.g. ethanol, lactic acid and acetic acid.

Glycolysis is a major catabolic pathway for degradation of carbohydrates in both aerobically and anaerobically growing microbial cells. The major input to glycolysis is glucose and the pathway, comprising a total of 10 different reactions, leads to the conversion of one molecule of glucose to two molecules of pyruvate, with the concomitant generation of ATP as well as a reduced form of a coenzyme termed nicotinamide adenine dinucleotide. The coenzyme may exist in either a reduced form, NADH, or an oxidised form, NAD^+.

For the glycolytic pathway to operate anaerobically, NADH must be reoxidised to NAD^+ by means of a transfer of electrons to a suitable electron acceptor so that a steady metabolic flux can be maintained. Microbial cells growing in the absence of oxygen have different ways of transferring such electrons. A simple route used by lactic acid bacteria

consists of simply using the synthesised NADH to reduce pyruvate to lactate, via the enzyme lactate dehydrogenase. NADH is reoxidised in the process:

$$\text{Pyruvate} + \text{NADH} \leftrightarrow \text{Lactate} + \text{NAD}^+$$

The lactic acid fermentation, i.e. conversion of glucose to lactic acid, is important in the manufacture of cheese. Another important fermentation involves a conversion of pyruvate to acetaldehyde and CO_2 and a reduction of acetaldehyde to ethanol mediated by alcohol dehydrogenase:

$$\text{Acetaldehyde} + \text{NADH} \leftrightarrow \text{Ethanol} + \text{NAD}^+$$

When carried out by yeasts, this fermentation generates the alcohol in alcoholic beverages. Yeasts used in baking also carry out this form of fermentation and the CO_2 produced by pyruvate decarboxylation causes bread to rise while the ethanol produced evaporates during baking. Among many other useful fermentations are those leading to e.g. acetic acid in the manufacture of vinegar and propionic acid in the manufacture of Swiss cheese.

Glycerol is another metabolic end product produced by numerous microbial cells. Glycerol is formed from dihydroxyacetone phosphate (DHAP) by a two-step reaction mediated by glycerol-3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase, respectively, as indicated below:

$$\text{DHAP} + \text{NADH} \rightarrow \text{Glycerol-3-Phosphate} + \text{NAD}^+$$

$$\text{Glycerol-3-Phosphate} \rightarrow \text{Glycerol} + \text{Phosphate}$$

Besides being involved in NADH reoxidation, glycerol formation in *Saccharomyces cerevisiae* is also involved in the generation of an effective osmolytic protection of the cell under stress conditions.

As described herein above, NADH must be reoxidised to NAD^+ by means of a transfer of electrons to a suitable electron for the glycolytic pathway to operate anaerobically. The reoxidised coenzyme in the form of NAD^+ contains a nicotinamide ring structure that is readily reducible and thus serves as an oxidising agent. Accordingly, after an enzymatic oxidation of a substrate such as an intermediate of the glycolytic pathway, the reduced form of the coenzyme, NADH, is dissociated from the enzyme and is reoxidised by any suitably available electron-acceptor system in the cell. The NAD^+ so formed is capable of repeating another cycle of coupled reduction and oxidation. NAD^+ and NADH thus differ from most substrates in that they are continually recycled.

A major source of electrons for reductive biosynthesis is NADPH, nicotinamide adenine dinucleotide phosphate. $NADP^+$ and NADPH are identical to NAD^+ and NADH, respectively, except that the form has an additional phosphate esterified at C-2′ on the adenylate moiety. NAD^+ and $NADP^+$ are equivalent in their thermodynamic tendency to accept electrons and they have similar standard reduction potentials. For reasons not known, nicotinamide nucleotide-linked enzymes that act in catabolic metabolism usually use the NAD^+/NADH coenzyme pair, whereas those acting in anabolic pathways tend to use $NADP^+$/NADPH. NADPH can be synthesised either from $NADP^+$ in the pentose phosphate pathway or from NADH through the action of an enzyme exhibiting a transhydrogenase activity.

The world ethanol production reached an estimated 31.3 billion litres in 1996. Approximately 80% were produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae*. Accordingly, ethanol is one of the most important biotechnological products with respect to both value and amount. Two thirds of the production is located in Brazil and in the United States with the primary objective of using ethanol as a renewable source of fuel. The demand and growth of this market is expected to give rise to a substantial growth in the ethanol production industry in the future. Hence, there are strong economic incentives to further improve the ethanol production process.

The price of the sugar source is a very important process parameter in determining the overall economy of ethanol production. Hence, it is of great interest to optimise the ethanol yield in order to ensure an efficient utilisation of the carbon source. Besides biomass

and carbon dioxide. a number of additional products are formed during anaerobic fermentation of *Saccharomyces cerevisiae* (Oura, 1977). Glycerol is the most dominant of these compounds, consuming up to 4% of the carbon source in industrial fermentations.

Accordingly, it is desirable to manipulate the metabolic flux in the direction from glycerol to ethanol and vice versa. When glycerol formation is both undesirable and a limiting factor in ethanol production, it is desirable to reduce and/or eliminate glycerol formation and to redirect the metabolic flux towards an increased production of ethanol. If successfully achieved, it should in theory be possible to increase the ethanol yield by a maximum of 4%, corresponding to an increase in the world production of ethanol of 1.25 billion litres per year without any additional costs. However. an increased production of glycerol may also be desirable. as glycerol is known to provide desirable organoleptic qualities in many wines. Accordingly, it is also desirable to reduce and/or eliminate formation of ethanol and to redirect the metabolic flux towards an increased production of glycerol.

SUMMARY OF THE INVENTION

The invention concerns a metabolic engineering of the capability of a microbial cell to produce one or more metabolic products such as e.g. ethanol, glycerol and lactic acid. Metabolic end products may either be produced concomitantly or the production of at least one metabolic end product may be increased while the production of additional end products is decreased accordingly. Increases and decreases in metabolic end product formation are guided by the metabolic potential of a cell and the flux of metabolites under certain metabolic conditions. Ethanol is a particularly preferred metabolic end product and is produced by a yeast cell under anaerobic conditions.

Although it is extremely difficult to alter or redirect a microbial metabolism, such as an anaerobic yeast metabolism, it may never the less be very desirable to alter a "traditional" profile of primary and/or secondary metabolites in order to achieve a different composition or "product mix", or in order to increase or decrease or even eliminate the production of some metabolites present in said profile.

It has now surprisingly been shown that the introduction into a yeast cell of an enzyme activity involved in oxidation and/or reduction of different coenzymes in a cell leads to a redirection of a metabolic flux in said cell.

In a first aspect there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity.

In another aspect there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said expression of said first expressible enzyme activity in said microbial cell being either novel to said cell or altered as compared to the expression of said first enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell.

In yet another aspect there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity, said expression of said first expressible enzyme activity in said microbial cell being either novel to said cell or altered as compared to the expression of said first enzyme directed by an expression signal natively associated with said first expressible enzyme activity.

In a further aspect there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity, said expression of said first expressible enzyme activity being operably linked to an increased production of a first metabolite.

In an even further aspect there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity, said expression of said first expressible enzyme activity being operably linked to an increased production of a first metabolite and a decreased production of a second metabolite.

Preferably, the microbial cell according to the invention as described herein above also comprises a further expressible enzyme activity, said further expressible enzyme activity, when expressed, mediates a first biosynthetic reaction resulting in a production of a first metabolite, said further expressible enzyme activity, when expressed at an increased level, results in an increased production of said first metabolite, said increased expression of said further expressible enzyme activity and/or said increased production of said first metabolite is operably linked to an increased expression of a first expressible enzyme activity controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity.

In yet another aspect there is provided a microbial cell comprising a further expressible enzyme activity, said further expressible enzyme activity, when expressed, mediates a first biosynthetic reaction resulting in a production of a first metabolite, said further expressible enzyme activity, when expressed at an increased level, results in an increased production of said first metabolite, said increased expression of said further expressible enzyme activity and/or said increased production of said first metabolite is operably linked to an increased expression of a first expressible enzyme activity resulting in maintenance and/or alteration of an intracellular redox system of said cell, said expression of said first enzyme activity in said microbial cell being either novel or altered as compared to the expression of said first enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell.

Other aspects of the invention provide a composition comprising a microbial cell and a physiologically acceptable carrier, a nucleotide sequence encoding a transhydrogenase enzyme activity, a recombinant replicon in the form of a vector harbouring said nucleotide sequence, a microbial cell transformed with said nucleotide sequence or said vector, and an amino acid sequence being encoded by said nucleotide sequence.

In yet another aspect there is provided a microbial cell or a composition for use in a production of a first metabolite or in a preparation of a drinkable or an edible product. In a further aspect there is provided a microbial cell or a composition for use in a method of generating an alternative intracellular NADH or NADPH reoxidation.

There is also provided the use of a microbial cell or a composition in a production of a first metabolite or in a preparation of a drinkable or an edible product. In another aspect there is provided the use of a microbial cell or a composition in a method of generating an alternative intracellular NADH or NADPH reoxidation.

In a further aspect there is provided a method of producing a first metabolite, said method comprising the steps of

i) cultivating a microbial cell in a suitable growth medium and under such conditions that said microbial cell is producing a first metabolite, and optionally

ii) isolating said first metabolite in a suitable form, and further optionally

iii) purifying said isolated first metabolite.

There is also provided a method of constructing a microbial cell, said method comprising introducing into said cell a capability and/or increasing a capability of said cell to express a first expressible enzyme activity which, when expressed in said microbial cell, is controlling a redox system of said cell, said first expressible enzyme activity being operably linked to an expression signal not natively associated with said activity.

In another aspect there is provided a method of constructing a microbial cell, said method comprising the steps of

i) operably linking a nucleotide sequence encoding a transhydrogenase activity with an expression signal not natively associated with said nucleotide sequence, and optionally

ii) operably linking a nucleotide sequence encoding a glutamate synthase activity with an expression signal not natively associated with said nucleotide sequence, and further optionally

iii) operably linking a nucleotide sequence encoding a glutamine synthetase activity with an expression signal not natively associated with said nucleotide sequence, and even further optionally

iv) reducing the expression of and/or eliminating a nucleotide sequence encoding a glutamate dehydrogenase activity in said cell, and

v) introducing said operably linked nucleotide sequence obtained under i), and optionally under ii) and iii), into a microbial cell, optionally the microbial cell obtained under iv), wherein said expression signal is expressible and, when expressed, results in expression of said operably linked nucleotide sequence.

DETAILED DESCRIPTION OF THE INVENTION

The invention concerns a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell. In one preferred embodiment, the expressible enzyme activity is a transhydrogenase activity the expression of which in a microbial cell is resulting in a modified i.e. redirected carbon flux or a redirected metabolic flux resulting at least in an increased production of a first metabolite and/or in a decreased production of a second metabolite.

In a microbial cell glucose is metabolised and biomass and metabolites are generated under i) an oxidation of NADPH to NADP and ii) a reduction of NAD to NADH. NADPH is regenerated e.g. when NADP is reduced in the pentose phosphate pathway. Conversion of pyruvate via acetaldehyde to acetate also contributes to a reduction of NADP and NADPH regeneration.

Oxidation of NADH and regeneration of a pool of NAD is occurring under anaerobic conditions by conversion of dihydroxy acetone phosphate (DHAP) via glycerol-3-phosphate to glycerol in a two-step reaction being mediated by glycerol-3-phosphat dehydrogenase and glycerol-3-phosphat phosphatase.

Consequently, it would be desirable to provide a microbial cell, preferably a yeast cell or a lactic acid bacterial cell, capable of performing NADH reoxidation without a concomitant generation of glycerol. Such an alternative reoxidation of NADH would in principle make it possible to direct a majority of an available carbon source into the production of ethanol without any significant production of - in this context - an undesirable "waste product" such as glycerol.

NADH reoxidation is essential to the microbial cell, as the synthesis, under anaerobic conditions, of biomass and an extensive and diverse range of reducible metabolites such as organic acids such as e.g. succinate, acetate, pyruvate and the like, results in a net formation of intracellular NADH which evidently cannot be reoxidised by means of the respiratory chain, as the respiratory chain is not functioning under anaerobic conditions. Intracellular NADH formation must therefore be balanced by a mechanism whereby NADH is reoxidised to NAD^+ in order to avoid depletion of the NADH pool under anaerobic conditions. Instead, NADH may be reoxidised to NAD^+ via the conversion of organic acids and/or aldehydes to organic alcohols such as e.g. glycerol, ethanol, propanol and/or lactic acid, since synthesis of these reduced metabolites leads to reoxidation of NADH and alleviates NADH depletion.

The process of intracellular interconversion of NAD^+ and NADH such as e.g. reoxidation of NADH generated during glycolysis occurs in an extremely complex environment wherein factors such as e.g. thermodynamics, metabolic flux, intricate allosteric regula-

tion mechanisms, and interconnected anabolic and/or catabolic metabolic pathways often makes it quite difficult for the skilled artisan to have any idea as to what will be the effect of a metabolic engineering of a redox level on the rate of synthesis of preferred metabolites or the overall yield of said metabolites. Accordingly, the person skilled in the art will have to formulate a hypothesis, which can be tested under reproducible conditions in order to verify or reject said hypothesis.

Often a hypothesis will have to be revised or even partially redrafted several times before it adequately and/or sufficiently accurately may describe e.g. i) a cellular metabolism or part thereof , ii) a metabolic flux or the flux of a carbon source in a cell, iii) an intricate allosteric regulation mechanism and its effect on e.g. enzyme activity and/or the rate of metabolite synthesis and/or product yield, or iv) a set of interconnected anabolic and/or catabolic metabolic pathways and changes therein caused e.g. by a change in the metabolic flux, which may in turn be caused by subjecting said cell to metabolical engineering.

The person skilled in the art will be aware of the pathways leading to the synthesis of many microbial primary and secondary metabolites and he will have access to relevant reference collections on the subject such as the authoritative Bergeys Manual. The person skilled in the art will also have to his disposal general biochemistry and molecular biology textbooks comprising state of the art insights into the complex world of cellular metabolism and biochemistry. Accordingly, even though the skilled artisan is no doubt confronted by a certain unpredictability in the art, he will never the less be able to deal with said unpredictability by inventing a sound and well researched strategy for subjecting a microbial cell to metabolic engineering.

In one presently preferred hypothesis, it is suggested that if NADH should conceivably be consumed by a transhydrogenase activity concomitantly with a conceivably increased production of NADPH, expression of a transhydrogenase gene encoding an expressible transhydrogenase activity, would most likely result in a decreased glycerol formation as well as a decreased carbon and/or metabolite flux through the pentose phosphate pathway involved in the production of carbon dioxide among others. Accordingly, a reduction in

the carbon flux towards undesirable "waste" components should be able to facilitate a redirection of carbon and/or metabolite flux towards an increased formation of ethanol.

Accordingly, there is provided a microbial cell comprising a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity.

The expression of said first expressible enzyme activity is either novel to said cell or altered as compared to the expression of said first enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell. Said expression of said first expressible enzyme activity is preferably operably linked to an increased production of a first metabolite. In one preferred embodiment, the cell comprises a further expressible enzyme activity, said further expressible enzyme activity, when expressed, mediating a first biosynthetic reaction resulting in production of a first metabolite, said further expressible enzyme activity, when expressed at an increased level, resulting in an increased production of said first metabolite, said increased expression of said further expressible enzyme activity and/or said increased production of said first metabolite being operably linked to an increased expression of said first expressible enzyme activity. There is also provided a microbial cell wherein said expression of said first expressible enzyme activity is operably linked to an increased production of a first metabolite and a decreased production of a second metabolite.

A comparable wild-type microbial cell or a comparable isolated microbial cell is a cell of the same species, preferably the same subspecies, as that of the microbial cell according to the invention.

The expressible enzyme activity is preferably operably linked to an expression signal not natively associated with said enzyme activity. When the expression of said first expressible enzyme activity is directed by an expression signal not natively associated with said activity, an altered expression will be understood to comprise any expression which is

altered as compared to the expression when being directed by an expression signal natively associated with said activity in a natural host organism.

The microbial cell in question may thus be a microbial eukaryote or a microbial prokaryote. Among microbial eukaryotes are many yeast and fungal cells preferred, such as yeast cells of the species *Saccharomyces*, *Schizosaccharomyces* and *Pichia*, such as e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and the like, as well as algae such as e.g. *Chlamydomonas reinhardi*, slime moulds such as e.g. *Dictyostelium discoideum* and filamentous fungi. Preferred filamentous fungi are species of *Neurospora* such as e.g. *Neurospora crassa*, and species of *Aspergillus* such as *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* species such as e.g. *Penicillium chrysogenum*, *Penicillium roqueforti* and *Penicillium camemberti*. Particularly preferred are industrially relevant yeast cells, slime moulds and filamentous fungi providing production of products such as e.g. antibiotics, steroids, pigments, enzymes, organic alcohols and acids, amino acids, polysaccharides and the like.

Among preferred microbial prokaryotes are bacterial cells such as Gram-positive species such as *Bacillus* species like e.g. *Bacillus subtilis*, *Bacillus thuringensis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*. *Bacillus cereus*, *Bacillus lentus* and *Bacilus stearothermophilus*, species of *Corynebacterium* like e.g. *Corynebacterium glutamicum*, and species of *Propionibacterium* as well as Gram-negative species such as *Escherichia coli*. Particularly preferred are also lactic acid bacterial species such as e.g. *Lactococcus lactis*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. diacetylactis*, *Leuconostoc* species such as e.g. *Leuconostoc oenos*, *Lactobacillus* species, such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus delbrückii subsp. bulgaricus*, and *Lactobacillus helveticus*, *Pediococcus* species, *Brevibacterium* species, *Propionibacterium* species and similar industrially relevant species like e.g. *Bifidobacterium*.

A particularly preferred microbial cell according to the invention is deposited with the DSM under Accession Numbers 12267 as *Saccharomyces cerevisiae* strain TN4.

An altered expression of said first expressible enzyme activity in a microbial cell according to the invention shall be understood to comprise any expression that differs with respect to the rate of product formation or with respect to the amount of product formed as compared to a comparable microbial cell. Accordingly, if a wild-type microbial cell is subjected to the metabolic engineering manipulations according to the invention, the skilled person will compare the expression of said first expressible enzyme activities provided in the metabolically engineered cell with the expression of the same activities in the wild-type microbial cell.

Generally, the person skilled in the art will preferably analyse - and compare with one another - similar or near identical microbial cells such as identical cells with and without a first expressible enzyme activity according to the invention. This is standard laboratory practise and the person skilled in the art will know how to conduct such an analysis so that it may form a basis for a direct comparison of e.g. an expressed enzyme activity or an expressed coenzyme or an expressed redox system within the meaning of those terms as set out herein below.

Preferably, the person skilled in the art will want to compare microbial cells to cells of at least the same species and more preferably to compare said cells to cells of at least the same subspecies.

Accordingly, if an isolated microbial cell such as e.g. an industrial strain or a strain in a culture collection is subjected to the metabolic engineering manipulations according to the invention, the skilled person will compare the expression of said first expressible enzyme activity provided in the metabolically engineered cell with the expression of the same activity in the industrial strain or the microbial cell from the culture collection.

The skilled artisan will know how to culture comparable strains such as strains of the same species or subspecies under identical or substantially similar conditions so as to provide a basis for performing the comparison between the relevant enzyme activities. The person skilled in the art will also know how to perform an enzymatic assay for use in said comparison and being indicative of the formation of e.g. a biosynthetic product resulting directly or indirectly from the action of said first expressible activity, when ex-

pressed, and he will be aware of the potential of transcriptional and/or translational fusions in monitoring expression of said expressible enzyme activities under comparable conditions. The skilled person will also be able to perform immunoassays including quantitative immunoprecipitations. An analysis of gene expression is available in e.g. Old and Primrose (1985): Principles of Gene Manipulation – An introduction to genetic engineering (Third edition), Blackwell Scientific Publications, Oxford, England.

The altered expression of said first expressible enzyme activity in a microbial cell, preferably a yeast cell or a lactic acid bacterial cell, shall preferably be understood to comprise an increased expression. Accordingly, the expression of said first expressible enzyme activity is increased by a factor of at least 1.02, such as a factor of at least 1.04, for example 1.06, such as 1.08, for example 1.10, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8, such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example an increase of at least 100.

In another embodiment, an increased expression of said first expressible enzyme activity in the microbial cell according to the invention is operably linked to an increased expression of a further expressible enzyme activity, said increased expression of said further activity is increased by a factor of at least 1.02, such as a factor of at least 1.04, for example 1.06, such as 1.08, for example 1.10, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8, such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example increased by a factor of at least 100.

In yet another embodiment, an increased expression of said first expressible enzyme activity in the microbial cell according to the invention is operably linked to an increased expression of a further expressible enzyme activity, said increased expression of said further activity results in an increased production of a first metabolite, said increased production of said first metabolite is increased by a factor of at least 1.02, such as a factor of at least 1.04, for example 1.06, such as 1.08, for example 1.10, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8, such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example increased by a factor of at least 100.

The first metabolite is preferably a primary or secondary metabolite and more preferably an amino acid, an alcohol or an acid, such as e.g. glutamat, lysin, threonin, aspartate, ethanol, glycerol, acetic acid, propionic acid. Ethanol and glycerol are particularly preferred. When the first metabolite is a secondary metabolite, said secondary metabolite is preferably selected from the group of secondary metabolites consisting of a β-lactam, a polyketide, a terpene, a steroid, a quinone, a coumarin, a flavonoid, an alkaloid, a piperidine, a pyridine, and the like.

However, an altered expression of said first expressible activity shall not be limited to an increased expression. A reduced expression of said first expressible activities shall also be understood to be comprised by the term altered expression. In a particularly preferred embodiment of the invention, said first expressible enzyme activity is one encoded by a nucleotide sequence designated SEQ ID NO:1, as illustrated herein below, or a functional derivative of said nucleotide sequence.

The terms altered expression and comparable microbial cell as introduced herein above apply to both of said first and further expressible enzyme activities. The terms redox system and intracellular redox system shall be understood to comprise any redox system comprising a coenzyme that is present in corresponding oxidised and reduced forms. Pre-

ferred intracellular redox systems are coenzymes in corresponding oxidised/reduced forms such as e.g. NAD/NADH, NADP/NADPH and FAD/$FADH_2$.

Any redox system can generally be perceived to contribute to the provision of a certain redox level in a cell. The totality of all such redox systems in a cell determines the redox level of said cell. The redox level of a cell thus comprises the presence and/or amount of the totality of reducing equivalents and oxidising equivalents present in said cell. An alteration of an intracellular redox system can be measured either by monitoring the increase or decrease of a specific redox system, i.e. an increase or decrease in both the oxidised form as well as in the reduced form of a coenzyme constituting said redox system, or alternatively, said alteration can be monitored by measuring an overall cellular redox level.

Accordingly, when it is desirable to maintain a certain redox level and prevent any increase or decrease in said level, the first expressible enzymatic activity is expressed and results in maintaining said desirable level. Accordingly, when it is desirable to increase a cellular redox level from an undesirable low level to a desirable higher level, said first expressible enzymatic activity is expressed and results in such an increase. Also, when it is desirable to decrease a cellular redox level from an undesirable high level to a desirable lower level, said first expressible enzymatic activity is expressed and results in such a decrease. It will be understood that redox level means both the cellular koncentration of an entire redox system, i.e. the koncentration of the corresponding reduced and oxidised forms of a coenzyme, as well as the cellular koncentration of either an oxidised or a reduced form of coenzyme. Accordingly, the first expressible enzymatic activity either maintains a redox level or promotes changes in said level. By expression of said first expressible enzymatic activity it is possible to regulate the redox level of a cell and promote the synthesis of desirable metabolites by providing a suitable redox level facilitating said synthesis or by e.g. increasing the intracellular yield of a coenzyme essential for the formation of said desirable metabolites.

The term maintenance of an intracellular redox system shall be understood to comprise the action exerted by a first expressible enzymatic activity which, when expressed, is acting e.g. in a pathway leading to the synthesis of one or more components of said sys-

tem or by acting in a recycling reaction or indeed in any cyclical reaction involving such components, preferably a reaction involving an oxidisation of a reduced coenzyme and/or a reduction of an oxidised coenzyme. It will be understood that maintenance and/or alteration involves controlling the redox level of a cell by increasing or decreasing the formation of an oxidised or reduced coenzyme such as e.g. a coenzyme belonging to the NAD/NADH, NADP/NADPH and FAD/$FADH_2$ redox systems. Said control will be understood to comprise e.g. maintaining a redox level in the cell, when said level would have been either reduced or increased in the absence of expression of said first expressible enzymatic activity.

Controlling the redox level will also be understood to comprise the provision of a desirable alteration i.e. a decrease or an increase of redox level in the cell, said alteration would have occurred in the absence of expression of said first expressible enzymatic activity. By increasing or decreasing the rate of synthesis and/or the pool of a redox system component, the first expressible enzyme activity is facilitating an alteration of said intracellular redox system. Accordingly, the terms increase and decrease relate to a level of expression or synthesis or to a concentration of a coenzyme and/or a redox system. The term level is used interchangeably in the art with terms such a synthesis rate and concentration. The person skilled in the art will be familiar with such terms and attach the correct meaning to their use in different contexts.

By exerting any one of the above-mentioned actions, said first expressible enzyme activity is controlling the maintenance of a redox system. Maintenance also comprises maintaining a redox level under conditions wherein said level would have been substantially decreased or increased had the cell not harboured said first expressible enzyme activity. The above-described maintenance of said redox system may well lead to an increased rate of synthesis of any one or more components of said system. Maintenance may also lead to an increase or decrease in the pool of any one component of said redox system, such as an increase or decrease of a reduced or oxidised form of a coenzyme.

The person skilled in the art will know how to assess an increase or decrease of any form of a coenzyme or of any redox system and he will know that he must compare the levels of that same coenzyme or redox system in a comparable wild-type microbial cell or an

isolated microbial cell grown under identical or substantially similar conditions that allow for a direct comparison of said levels by exploiting state of the art monitoring techniques such as those described by Weuster and de-Graff (1996) in Adv. Biochem. Eng. Biotechnol., vol. 54, pages 75 – 108 and by Wiechert and de-Graff (1996) in Adv. Biochem. Eng. Biotechnol., vol. 54, pages 109 – 154. The person skilled in the art will preferably analyse similar or substantially identical microbial cells with and without said first expressible enzyme activity. Similar or substantially identical microbial cells are e.g. cells of the same species or the same subspecies.

In one embodiment, when said first expressible enzyme activity is expressed, the expression results in an increased or decreased level, preferably an increased level, of at least one intracellular coenzyme in its oxidised or reduced form. Said coenzyme in its oxidised/reduced form is preferably selected from the group consisting of FAD/$FADH_2$, NAD/NADH and NADP/NADPH.

Accordingly, the level of at least one intracellular coenzyme in its oxidised or reduced form is either increased or decreased, preferably increased, such as increased by a factor of at least 1.005, for example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1,065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085, for example a factor of at least 1.090, such as 1.095, for example 1.100, such as 1.105, for example 1.110, such as 1.115, for example 1.120, such as at least 1,125, for example a factor of at least 1.130, such as 1.135, for example 1.140, for example 1.145, such as 1.150, for example 1.155, such as at least 1.160, for example a factor of at least 1.165, such as a factor of at least 1.170, for example a factor of at least 1.175, such as 1.180, for example 1.185, such as 1.190, for example 1.195, such as a factor of at least 1.200, for example 1.21, such as 1.22, for example 1.23, such as 1.24, for example 1.25, such as 1.26, for example 1.27, such as 1.28, for example 1.29, such as a factor of at least 1.30, for example 1.35, such as 1.40, for example 1.45, such as 1.50, for example 1.55, such as 1.60, for example 1.65, such as 1.70, for example 1.75, such as 1.80, for example 1.85, such as 1.90, for example 1.95, such as a factor of at least 2.0, for example 2.2, such as 2.4, for example 2.6, such as 2.8, for example 3.0, such as 3.2, for example 3.4,

such as 3.6, for example 3.8, such as at least 4.0, for example 4.2, such as 4.4, for example 4.6, such as 4.8, for example at least 5.0, such as 5.2, for example 5.4, such as 5.6, for example 5.8, such as at least 6.0, for example 6.2, such as 6.4, for example 6.6, such as 6.8, for example at least 7.0, for example 7.2, such as 7.4, for example 7.6, such as 7.8, for example 8.0, such as 8.2, for example 8.4, such as 8.6, for example 8.8, such as at least 9.0, for example 9.2, such as 9.4, for example 9.6, such as 9.8, for example a factor of at least 10.0.

Although an increase is preferred, it shall be understood that the term alteration is by no means limited to an increase in the level of at least one intracellular coenzyme in its oxidised or reduced form. Said alteration shall also comprise any decrease in the level of at least one intracellular coenzyme in its oxidised or reduced form.

In an embodiment of the invention wherein the expression of said first expressible enzyme activity results in an increase or a decrease, preferably an increase, in the level i.e. concentration of an intracellular redox system, i.e. an increase or decrease, preferably an increase, of both of two corresponding oxidised and reduced forms of a coenzyme, such as an increase by a factor of at least 1.005, for example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1.065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085. for example a factor of at least 1.090, such as 1.095, for example 1.100, such as 1.105, for example 1.110, such as 1.115, for example 1.120, such as at least 1,125, for example a factor of at least 1.130, such as 1.135, for example 1.140, for example 1.145, such as 1.150, for example 1.155, such as at least 1.160, for example a factor of at least 1.165, such as a factor of at least 1.170, for example a factor of at least 1.175, such as 1.180, for example 1.185, such as 1.190, for example 1.195, such as a factor of at least 1.200, for example 1.21, such as 1.22, for example 1.23, such as 1.24, for example 1.25, such as 1.26, for example 1.27, such as 1.28, for example 1.29, such as a factor of at least 1.30, for example 1.35, such as 1.40, for example 1.45, such as 1.50, for example 1.55, such as 1.60, for example 1.65, such as 1.70, for example 1.75, such as 1.80, for example 1.85, such as 1.90, for example 1.95, such as a factor of at least 2.0, for example 2.2, such as 2.4, for example 2.6, such as 2.8, for example 3.0, such as 3.2, for

example 3.4, such as 3.6, for example 3.8, such as at least 4.0, for example 4.2, such as 4.4, for example 4.6, such as 4.8, for example at least 5.0, such as 5.2, for example 5.4, such as 5.6, for example 5.8, such as at least 6.0, for example 6.2, such as 6.4, for example 6.6, such as 6.8, for example at least 7.0, for example 7.2, such as 7.4, for example 7.6, such as 7.8, for example 8.0, such as 8.2, for example 8.4, such as 8.6, for example 8.8, such as at least 9.0, for example 9.2, such as 9.4, for example 9.6, such as 9.8, for example a factor of at least 10.0.

Said first expressible enzyme activity may well result in an increase or a decrease, preferably an increase, of more than one intracellular redox system. It shall be understood that in one embodiment of the invention, said first expressible enzyme activity, when expressed, is resulting in an increased level of at least one intracellular redox system.

In a particularly preferred aspect of the invention, said first expressible enzyme activity is an intracellular transhydrogenase activity, preferably a pyridine nucleotide transhydrogenase activity, and even more preferably the pyridine nucleotide transhydrogenase activity of *CTH* of *Azotobacter vinelandii* as harboured by *Saccharomyces cerevisiae* TN4 as deposited under DSM Accession Number 12267, or a functionally equivalent activity. A functionally equivalent activity is any activity capable of carrying out a substantially similar reaction with the provision of a similar outcome as that resulting from the reaction being carried out by the above-mentioned *CTH* encoded polypeptide when harboured by *Saccharomyces cerevisiae*.

The pyridine nucleotide transhydrogenase activity is either endogenous or heterologous to said microbial cell, and said activity is either exhibited by a polypeptide which is membrane-bound in a natural host organism or located i.e. present in the cytoplasm of a natural host organism, said natural host organism preferably being selected from the group of mammalian cells, eukaryotic cells including microbial eukaryotic cells, and prokaryotic microbial cells, preferably a Gram-positive microbial prokaryote or a Gram-negative microbial prokaryote.

Expression of said pyridine nucleotide transhydrogenase activity in said microbial cell in one preferred embodiment of the invention results in an increased conversion of NADPH

and NAD to NADH and NADP. In another embodiment said expression of said pyridine nucleotide transhydrogenase activity results in an increased consumption of NADPH and an increased formation of NADH. In another embodiment the expression has the effect of increasing the consumption of NADH and increasing the formation of NADPH. In yet another embodiment the expression results in an increased formation of NADH and/or NADP. However, said expression may also result in a decreased formation of NADPH and/or a decreased NADPH/NADP retio. The pyridine nucleotide transhydrogenase activity is preferably expressed in said microbial cell under anaerobic growth conditions and preferably results in the ratio of NADPH/NADP being lower than the ratio of NADH/NAD.

In another preferred embodiment, the transhydrogenase activity, preferably an activity that is membrane-bound in a natural host organism, is inserted into the plasma membrane of a microbial cell, preferably a yeast cell. The transhydrogenase activity mediates a reaction consuming NADP and generating NADPH. In one preferred embodiment, an *E. coli* transhydrogenase is expressed in a yeast cell and leads to an increased level of NADPH. The expression of the yeast plasma membrane located *E. coli* transhydrogenase is coupled to a proton gradient across said membrane similar to the coupling observed in a natural host organism i.e. *E. coli*.

The microbial cell according to invention is preferably one suitable for storage in the form of a frozen or freeze-dried preparation such as a lyophilisate from which the microbial cell is partly or wholly reconstitutable.

In yet another embodiment of the invention, the microbial cell has been metabolically engineered as described above and is capable of alternative NADH re-oxidation. Said alternative NADH re-oxidation is mediated at least by expression of said first expressible enzyme activity and optionally also by expression of a further expressible enzyme activity. Alternative NADH re-oxidation shall be understood to comprise the introduction of a novel major pathway for NADH re-oxidation or a generation of a substantially altered pathway for NADH-reoxidation in a microbial cell. Alteration in respect of a pathway for alternative NADH oxidation shall be understood in the context of the rate of a reaction mediating a conversion of one metabolite to another, said reaction also resulting in

NADH re-oxidation. The rate of said re-oxidation reaction in a microbial cell capable of alternative NADH re-oxidation is substantially increased as compared to the rate of said reaction in a comparable microbial cell. The definition of the term comparable in respect of microbial cells is introduced herein above.

Alternative NADH re-oxidation in one example is a microbial cell wherein the expression of a transhydrogenase activity, in combination with several additionally preferred expressible enzyme activities, is capable of generating a purposeful redesigning of a complex network of metabolic reactions. The redesigned microbial cell is invented by replacing or supplementing a normally dominant first metabolic reaction with a second reaction that is normally insignificant in relation to reaction rate and/or product formation as compared to said first dominant reaction. However, by significantly increasing said second reaction while at the same time significantly decreasing or even eliminating said first reaction, it is possible to achieve an alternative NADH re-oxidation. In a further embodiment of the invention, there is also provided a microbial cell capable of alternative NADPH re-oxidation or alternative NADP reduction.

In another aspect of the invention, there is provided a composition comprising the microbial cell and a carrier, preferably a physiologically acceptable carrier and more preferably a water-based liquid such as a broth suitable for culturing said microbial cell. The composition in a preferred embodiment is a fermentation starter culture.

There is also provided the aspect of a nucleotide sequence encoding a novel and industrially relevant transhydrogenase enzyme activity, said sequence comprising SEQ ID NO:1 as illustrated herein below, or part thereof, including functionally equivalent derivatives thereof encoding a transhydrogenase enzyme activity, preferably but not limited to conservative nucleotide substitutions and/or nucleotide deletions and/or nucleotide insertions.

Said functionally equivalent derivatives may thus be at least 99.9 percent identical to SEQ ID NO:1, such as at least 99.8 percent identical to SEQ ID NO:1, for example at least 99.7 percent identical, such as at least 99.6 percent identical, , for example at least 99.5 percent identical, such as at least 99.4 percent identical, for example at least 99.3

percent identical, such as at least 99.2 percent identical, for example at least 99.1 percent identical, such as at least 99 percent identical to SEQ ID NO:1, for example at least 98.5 percent identical to SEQ ID NO:1, such as at least 98.0 percent identical, for example 97.5 percent identical, such as at least 97.0 percent identical to SEQ ID NO:1, for example at least 96.5 percent identical, such as at least 96.0 percent identical, for example at least 95.5 percent identical, such as at least 95.0 percent identical, for example at least 94.5 percent identical, such as at least 94.0 percent identical, for example at least 93.5 percent identical, such as at least 93.0 percent identical to SEQ ID NO:1, for example at least 92.5 percent identical, such as at least 92.0 percent identical, for example at least 91.5 percent identical, such as at least 91.0 percent identical, for example at least 90.5 percent identical, such as at least 90.0 percent identical, for example at least 85.0 percent identical to SEQ ID NO:1. In one embodiment, SEQ ID NO:1 is a sequence that is synthesised partly or wholly *in vitro*.

Functionally equivalent derivatives may have an altered nucleotide sequence and may encode a polypeptide having an altered amino acid sequence as compared to that encoded by SEQ ID NO:1. However, said polypeptides are characterised by a similar or substantially identical enzymatic activity and are thus functionally equivalent.

In one embodiment, the functionally equivalent nucleotide sequence is provided by means of homologous or semi-homologous recombination of identical or substantially identical nucleotide sequences being part of different genes and located e.g. on the same or different extrachromosomal replicons. The person skilled in the art will be familiar with the construction of hybrid genes in this way. Accordingly, in one embodiment, the transhydrogenase gene according to the invention is composed of nucleotide sequences originating from different genes such as e.g. different genes encoding a cytoplasmatically located transhydrogenase and/or a membrane-bound transhydrogenase. This technique is also termed gene-shuffling in the art.

The nucleotide sequence, when being expressed in a microbial cell, is preferably operably linked to an expression signal not natively associated with said nucleotide sequence. The expression signal is preferably one generating at least a substantially constitutive expression, such as the *PGK* promoter, an inducible expression signal such as a growth

phase regulated promoter or a promoter induced by e.g. pH or temperature and changes therein.

The expression signal is preferably a regulable expression signal such as a regulable transcription initiation signal and/or a regulable translational initiation signal, such as an expression signal regulatable in response to an alteration in a value, level and/or concentration of a factor such as a physiological growth parameter, preferably a parameter selected from the group consisting of pH, temperature, salt content including osmolarity, anaerobicity, aerobicity including oxygen level, energy level including a membrane potential and a proton motive force.

The promoter is preferably a promoter being either growth phase regulated, inducible and/or repressible and/or, in a natural host organism, directing expression of a gene encoding a gene product involved in mediating a reaction of a biosynthetic pathway and/or a major metabolic pathway, preferably a pathway selected from the group of pathways consisting of glycolysis, gluconeogenesis, citric acid cycle, and pentose phosphate pathway.

The expression signal may be further regulated by an upstream activating sequence (UAS), by an enhancer element or by a silencer element. The person skilled in the art will be aware of general molecular biology techniques for use in the construction in vitro of a recombinant DNA molecule. Such techniques are described e.g. in Sambrook et al. (1989) and in Old and Primrose (ibid.). Said skilled artisan will further be aware of the academic literature including general textbooks on molecular biology and genetic engineering and he will be able to combine various expression signals such as putative or recognised promoter regions with a range of regulatory nucleotide sequences generally known to exert an effect on the level of gene expression. The skilled person is able to monitor gene expression by construction of suitable transcriptional and/or translational fusions of an expression signal to a reporter gene generally available in the art. An expression signal can be a cloned expression signal or an in vitro synthesised expression signal. Expression signals in prokaryotic microbial cells are known to comprise so-called –35 and –10 regions and numerous examples of such regions are available from various databases.

Expression signals may be optimised by increasing the promoter strength, by adjusting translational initiation sequences, by optimising the choice of codons by using so-called highly expressed codons, by adjusting the secondary structure of the mRNA, by increasing the efficiency of transcriptional termination, by increasing or decreasing a copy number of a vector, or by increasing or decreasing the stability of said vector.

In a further aspect of the invention there is provided a recombinant DNA-replicon in the form of a vector comprising the nucleotide sequence designated SEQ ID NO:1 as illustrated herein above including functionally equivalent derivatives. The nucleotide sequence designated SEQ ID NO:1 is operably linked to an expression signal comprised in said replicon, said expression signal directing expression of said nucleotide sequence. There is also provided a microbial cell microbial cell, preferably a yeast cell, harbouring the nucleotide sequence designated SEQ ID NO:1 or a recombinant replicon in the form of a vector harbouring said nucleotide sequence.

The recombinant DNA-replicon is preferably one capable of replicating in a yeast cell and/or in a prokaryotic microbial cell such as a lactic acid bacterial cell. Preferred yeast vectors comprise a selectable marker and one or more sites in a nucleotide sequence specific for a restriction endonuclease. An autonomously replicating sequence (ARS) mediates replication of said replicon when harboured in a yeast cell. The vector is preferably based on a plasmid selected from the group consisting of a yeast episomal plasmid (Yep), such as the 2 μm plasmid, a yeast replicating plasmid (Yrp), a yeast centromeric plasmid (Ycp) and a yeast integrating plasmid (Yip). A particularly preferred replicon is the one harboured by *Saccharomyces cerevisiae* strain so and so as deposited under accession number DSM/ATCC number so and so.

Vectors capable of being maintained in a prokaryotic microbial cell such as a lactic acid bacterial cell are well described in the literature and preferably contain a replicon directing e.g. rolling circle replication or θ-replication, a selectable marker such as a nonsense mutation preventing selection and/or replication unless suppressed by a suppresser comprised by a cell comprising said vector, and one or more sites cleavable by a restriction endonuclease.

In a further aspect of the invention, there is provided an amino acid sequence encoded by the nucleotide sequence designated SEQ ID NO:1, or any functionally equivalent derivative thereof, said amino acid sequence comprising the sequence SEQ ID NO:2 as illustrated herein below, or a part thereof, including any functionally equivalent derivatives exhibiting transhydrogenase activity, and preferably, but not limited to, functionally equivalent derivatives comprising conservative amino acid substitutions. Functionally equivalent derivatives may have an altered amino acid sequence as compared to that encoded by SEQ ID NO:2. However, said polypeptides are characterised by a similar or substantially identical enzymatic activity and are thus functionally equivalent.

Said functionally equivalent derivative of said amino acid sequence designated SEQ ID NO:2 may thus be at least 99 percent identical to SEQ ID NO:2. such as at least 98 percent identical to SEQ ID NO:2, for example at least 97 percent identical, such as at least 96 percent identical. for example at least 95 percent identical. such as at least 94 percent identical, for example at least 93 percent identical, such as at least 92 percent identical, for example at least 91 percent identical, such as at least 90 percent identical to SEQ ID NO:2, for example at least 89 percent identical to SEQ ID NO:2, such as at least 88 percent identical, for example 87 percent identical, such as at least 86 percent identical to SEQ ID NO:2, for example at least 85 percent identical to SEQ ID NO:2. In one embodiment, SEQ ID NO:2 is a sequence that is synthesised partly or wholly *in vitro*.

The microbial cell according to the invention in one embodiment further comprises a second and a third expressible enzyme activity which, when independently expressed in said microbial cell, are facilitating or controlling assimilation in said cell of a nutrient source, preferably ammonia, said expression of said second and third expressible enzyme activities in said microbial cell being either novel or altered as compared to the expression of said second enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell, said second expressible enzyme activity being non-identical to said first expressible enzyme activity. Said second and/or third expressible enzyme activities are preferably operably linked to an expression signal such as a promoter not operably linked to said activities in a natural host organism. Said altered expression is an expression being different from that directed by an expression signal natively associated with said expressible activities in a natural host organism.

The nutrient source is capable of sustaining microbial growth by e.g. being assimilated into a biosynthetic product that can be utilised by a microbial cell or be converted i.e. metabolised into a further biosynthetic product, said utilisation and/or metabolism involving one or more energy yielding metabolic reactions. Accordingly, a nutrient source is any source that is potentially assimilable and metabolisable by a microbial cell. The step of assimilation shall be understood to comprise both uptake of said source into said call as well as conversion of said source into a biosynthetic product - an intermediate metabolite - within said cell. Preferably assimilation is the conversion that takes place within the cell without necessarily being limited to this step of the assimilation process.

Preferred assimilable nutrient sources comprise ammonia, ammonium ions, nitrite ions, and nitrate ions. The assimilable nitrogen source according to the invention is more preferably ammonia and ammonium ions and most preferably ammonia. It will be understood that the invention pertains to all nitrogen containing nutrient sources capable of being converted into ammonia by oxidation including biological oxidation or by reduction including biological reduction.

A biosynthetic reaction mediated by said second expressible enzyme activity, when expressed, is preferably a reaction capable of being carried out by action of a metabolite synthase enzyme, more preferably by an allosteric metabolite synthase enzyme, and even more preferably is said reaction carried out by an expressible enzyme activity which, when expressed, is exhibited by a glutamate synthase.

In a particularly preferred embodiment of the invention said glutamate synthase activity is that of *GLT1* of *Saccharomyces cerevisiae* TN17 as deposited under DSM Accession Number 12275, or an activity functionally equivalent therewith. A functionally equivalent activity is any activity capable of carrying out the same reaction with the provision of a similar outcome as that resulting from the reaction being carried out by the above-mentioned *GLT1* encoded polypeptide of *Saccharomyces cerevisiae*.

A biosynthetic reaction mediated by said third expressible enzyme activity, when expressed, is preferably a reaction capable of being carried out by action of a metabolite

synthetase enzyme, and more preferably is said reaction carried out by an expressible enzyme activity which, when expressed, is exhibited by a glutamine synthetase.

In a particularly preferred embodiment of the invention, said glutamine synthetase activity is that encoded by *GLN1* of *Saccharomyces cerevisiae* TN15 as deposited under DSM Accession Number 12274, or an activity functionally equivalent therewith. A functionally equivalent activity is any activity capable of carrying out the same reaction with the provision of a similar outcome as that resulting from the reaction being carried out by the above-mentioned *GLN1* encoded activity of *Saccharomyces cerevisiae*.

Accordingly, in a particularly preferred embodiment there is provided a microbial cell, preferably a yeast cell, wherein said second expressible enzyme activity is a metabolite synthase activity, more preferably an allosteric metabolite synthase activity, and even more preferably a glutamate synthase activity and wherein said third expressible enzyme activity is a metabolite synthetase activity, preferably a glutamine synthetase activity.

In another preferred embodiment, said second or third expressible enzyme activity a is ligase activity such as e.g. or a NADH-dependent glutamate dehydrogenase activity or a NADPH-dependent glutamate dehydrogenase activity.

In yet another embodiment there is provided a microbial cell comprising a reduced expression or no expression of a fourth expressible enzyme activity which, when being present in said cell, is operably linked to an expression signal not natively associated with said fourth activity, said fourth expressible enzyme activity facilitates or controls assimilation in said cell of a nutrient source, said expressible fourth enzyme activity may optionally be operably linked to an expression signal not natively associated with said activity, said fourth expressible enzyme activity being non-identical to each and both of said second and third expressible enzyme activities.

It is particularly preferred to delete said fourth expressible enzyme activity when the microbial cell is a yeast cell, but the activity may also be deleted from any other eukaryotic microbial cell or from a prokaryotic microbial cell. Reference is made to the above-mentioned definitions of terms such as assimilation, nutrient source, altered expression and

comparable microbial cell. The interpretations indicated herein above, when applicable, also apply with respect to said expressible fourth enzyme activity.

Accordingly, in one preferred embodiment according to the invention, the fourth expressible enzyme activity is preferably a metabolite dehydrogenase activity, and more preferably a glutamate dehydrogenase activity, and most preferably a NADPH-dependent glutamate dehydrogenase activity, which is either present in said microbial cell in a reduced amount, more preferably in a substantially reduced amount, or eliminated from said cell by means of e.g. deletion of a nucleotide sequence encoding said activity or by effectively repressing the expression of said expressible fourth enzyme activity.

Consequently, an altered expression in said microbial cell according to the invention of said fourth expressible enzyme activity, preferably a glutamate dehydrogenase activity, and even more preferably a NADPH-dependent glutamate dehydrogenase activity, shall be understood to comprise an expression reduced by at least 1 percent, such as decreased by at least 2 percent, for example 4 percent, such as 6 percent, such as at least 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, such as at least 18 percent, for example at least 20 percent, such as 22 percent, for example 24 percent, such as 26 percent, such as at least 28 percent, for example at least 30 percent, such as 32 percent, for example 34 percent, such as 36 percent, for example 38 percent, such as at least 40 percent, for example 42 percent, such as 44 percent, for example 46 percent, such as 48 percent, such as at least 50 percent, for example 52 percent, such as 54 percent, for example 56 percent, such as 58 percent, such as at least 60 percent, for example 62 percent, such as 64 percent, for example 66 percent, such as 68 percent, such as at least 70 percent, for example 72 percent, such as 74 percent, for example 76 percent, such as 78 percent, such as at least 80 percent, for example 82 percent, such as 82 percent, for example 84 percent, such as 86 percent, such as at least 88 percent, for example 90 percent, such as 92 percent, for example 94 percent, such as 96 percent, for example at least 98 percent, such as 99 percent, for example 99.2 percent, such as at least 99.4 percent, for example 99.6 percent, such as 99.8 percent, for example 99.9 percent, such as 99.92 percent, for example 99.94 percent, such as 99.96 percent, for example 99.98 percent, such as 99.99 percent, for example decreased to such an extend

that said expression is unassayable using standard state of the art assays and/or said expression is effectively repressed and/or substantially eliminated.

However, an altered expression shall not be limited to a decreased expression. An increased expression of said fourth expressible enzyme activity shall also be understood to be comprised by the term-altered expression.

In a particularly preferred embodiment of the invention, said glutamate dehydrogenase activity is that of a *GDH1* encoded polypeptide of *Saccharomyces cerevisiae* TN9 deposited with the DSM under Accession Number 12268, or an activity functionally equivalent therewith.

In a particularly preferred embodiment of the invention, there is provided a microbial cell wherein the expression of said second and third expressible enzyme activities is increased, preferably substantially increased, whereas the expression of said fourth expressible enzyme activity is decreased, preferably substantially decreased, or eliminated, such as the level of expression of said activities in e.g. *Saccharomyces cerevisiae* strain TN 19 deposited with DSM under Accession Number 12276.

A microbial cell according to the invention is preferably a yeast cell wherein the expression of said second and third expressible enzyme activities is increased, preferably substantially increased, whereas the expression of said fourth expressible enzyme activity is decreased, preferably substantially decreased, and said cell may in one embodiment produce a first metabolite, such as e.g. ethanol or glycerol, said production of said first metabolite is increased as compared to an expression of said metabolite in a comparable wild-type or isolated cell, said increase is increased by a factor of at least 1.005, for example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1,065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085, for example a factor of at least 1.090, such as 1.095, for example 1.100, such as 1.105, for example 1.110, such as 1.115, for example 1.120, such as at least 1,125, for example a factor of at least 1.130, such as 1.135, for example 1.140, for example 1.145, such as 1.150, for ex-

ample 1.155, such as at least 1.160, for example a factor of at least 1.165, such as a factor of at least 1.170, for example a factor of at least 1.175, such as 1.180, for example 1.185, such as 1.190, for example 1.195, such as a factor of at least 1.200, for example 1.21, such as 1.22, for example 1.23, such as 1.24, for example 1.25, such as 1.26, for example 1.27, such as 1.28, for example 1.29, such as a factor of at least 1.30, for example 1.35, such as 1.40, for example 1.45, such as 1.50, for example 1.55, such as 1.60, for example 1.65, such as 1.70, for example 1.75, such as 1.80, for example 1.85, such as 1.90, for example 1.95, such as a factor of at least 2.0, for example 2.2, such as 2.4, for example 2.6, such as 2.8, for example 3.0, such as 3.2, for example 3.4, such as 3.6, for example 3.8, such as at least 4.0, for example 4.2, such as 4.4, for example 4.6, such as 4.8, for example at least 5.0, such as 5.2, for example 5.4, such as 5.6, for example 5.8, such as at least 6.0, for example 6.2, such as 6.4, for example 6.6, such as 6.8, for example at least 7.0, for example 7.2, such as 7.4, for example 7.6, such as 7.8, for example 8.0, such as 8.2, for example 8.4, such as 8.6, for example 8.8, such as at least 9.0, for example 9.2, such as 9.4, for example 9.6, such as 9.8, for example a factor of at least 10.0.

Said microbial cell, most preferably yeast, having an increased production of a first metabolite has, in another preferred embodiment, a decreased production of a second metabolite, preferably glycerol or ethanol. Said decreased production of said second metabolite is decreased by at least 0.5 percent, for example at least 1 percent, such as at least 2 percent, for example 4 percent, such as 6 percent, such as at least 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, such as at least 18 percent, for example at least 20 percent, such as 22 percent, for example 24 percent, such as 26 percent, such as at least 28 percent, for example at least 30 percent, such as 32 percent, for example 34 percent, such as 36 percent, for example 38 percent, such as at least 40 percent, for example 42 percent, such as 44 percent, for example 46 percent, such as 48 percent, such as at least 50 percent, for example 52 percent, such as 54 percent, for example 56 percent, such as 58 percent, such as at least 60 percent, for example 62 percent, such as 64 percent, for example 66 percent, such as 68 percent, such as at least 70 percent, for example 72 percent, such as 74 percent, for example 76 percent, such as 78 percent, such as at least 80 percent, for example 82 percent, such as 82 percent, for example 84 percent, such as 86 percent, such as at least 88 percent, for

example 90 percent, such as 92 percent, for example 94 percent, such as 96 percent, for example at least 98 percent, such as 99 percent, for example 99.2 percent, such as at least 99.4 percent, for example 99.6 percent, such as 99.8 percent, for example 99.9 percent, such as 99.92 percent, for example 99.94 percent, such as 99.96 percent, for example 99.98 percent, such as 99.99 percent, for example an expression level being decreased to such an extend that said expression of said fourth activity is unassayable using standard state of the art assays and/or said expression is effectively repressed and/or substantially eliminated.

In another particularly preferred embodiment, the maximum specific growth rate of said cell according to the invention is substantially unaltered as compared to a comparable wild-type microbial cell or to a comparable isolated microbial cell. However, a microbial cell characterised by a decrease in the maximum specific growth rate is also preferred according to the invention, such as a microbial cell, preferably a yeast cell, having a maximum specific growth rate that is decreased by less than 1 percent, such as 1.5 percent, for example 2.0 percent, such as by less than 2.5 percent, for example 3.0 percent, such as 3.5 percent, for example by less than 4.0 percent, such as 4.5 percent, for example 5.0 percent, such as by less than 5.5 percent, for example 6.0 percent, such as 6.5 percent, for example by less than 7.0 percent, such as 7.5 percent, for example 8.0 percent, such as by less than 8.5 percent, for example 9.0 percent, such as 9.5 percent, for example by less than 10.0 percent, such as 12 percent, for example 14 percent, such as by less than 16 percent, for example 18 percent, such as 20 percent, for example by less than 25 percent.

In a further aspect of the invention there is provided a microbial cell, preferably a yeast cell or a bacterial cell, or a composition comprising said cell, for use in a production of a first metabolite such as a primary or secondary metabolite, preferably a primary metabolite and more preferably an amino acid, an alcohol or an acid, such as e.g. ethanol, glycerol, acetic acid and propionic acid, ethanol and/or glycerol being particularly preferred.

When the first metabolite is a secondary metabolite, said secondary metabolite is preferably selected from the group of secondary metabolites consisting of a β-lactam, a

polyketide, a terpene, a steroid, a quinone, a coumarin, a flavonoid, an alkaloid, a piperidine, a pyridine, and the like.

It may furthermore be advantageous to produce and/or purify by means of any state of the art down-stream processing technique said first metabolite in an organism such as e.g. a fungal cell, a yeast cell or a bacterial cell. Any of said eukaryotic or prokaryotic cells for use in said production preferably qualify for GRAS status ("Generally Regarded As Safe") with the Federal Drug Administration of the United States of America.

In another embodiment, there is provided a microbial cell, preferably a yeast cell, for use in said production of said first metabolite, said cell further producing a second metabolite, preferably glycerol or ethanol, said production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell.

When the microbial cell is a prokaryotic cell such as e.g. a lactic acid bacterial cell for use in the production of a first metabolite, said first metabolite is selected from the group consisting of lactic acid and an aroma component such as acetoin, acetaldehyde, diacetyl, and 2,3-butylene glycol.

When the microbial cell is a cell such as e.g. a *Bacillus* cell for use in the production of a first metabolite, said cell is capable of further producing e.g. a protease, an amylase, a cellulase, a β-glucanase, an endoglucanase, a phosphatase, a xylanase, a lipase, a β-lactamase, or a xylosidase.

In one embodiment, the microbial cell or the composition according to the invention is preferably used in a production of a first metabolite or used in a method of generating alternative intracellular NADH re-oxidation. Accordingly, the microbial cell is providing a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADH re-oxidation for the purpose of providing, supplementing and/or increasing a pool of intracellular NAD, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In another embodiment there is provided a microbial cell for use in a production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NAD reduction for the purpose of providing, supplementing and/or increasing a pool of intracellular NADH, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In yet another embodiment of the invention, there is provided a microbial cell for use in the production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADPH re-oxidation for the purpose of providing, supplementing and/or increasing a pool of intracellular NADP, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In a still further embodiment of the invention, there is provided a microbial cell for use in the production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADP reduction for the purpose of providing, supplementing and/or increasing a pool of intracellular NADPH, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In a yet further aspect of the invention there is provided a microbial cell according to invention for use in a preparation of a drinkable or an edible product. There is also provided a microbial cell for use in a production of a first metabolite for use in a drinkable or an edible product, preferably a product having desirable organoleptic qualities. In one embodiment, said first metabolite has and/or provides a desirable organoleptic quality to said product. In a particularly preferred embodiment, said first metabolite is ethanol.

The microbial cell for use in a production of a first metabolite according to the invention, in one embodiment, further produces a second metabolite, the production of said second metabolite being substantially decreased as compared to the production of said second

metabolite in a comparable wild-type cell or a comparable isolated microbial cell, said decreased production resulting in a provision of a desirable organoleptic quality to said product. In a further embodiment said product is a functional food.

In yet another aspect of the invention there is provided the use of a microbial cell or a composition comprising said cell in a production, preferably an increased production, of a first metabolite, said metabolite being a primary metabolite or a secondary metabolite. It is preferred that the microbial cell is a yeast cell or a prokaryotic microbial cell and that said first metabolite is an alcohol or an acid, preferably ethanol, glycerol, acetic acid, lactic acid or propionic acid. Further preferred metabolites are selected from the group consisting of a β-lactam, a polyketide, a terpene, a steroid, a quinone, a coumarin, a flavonoid, an alkaloid, a piperidine, a pyridine, and the like.

In a preferred use there is provided a microbial cell, preferably a yeast cell, further producing a second metabolite, said production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell. Particularly preferred is a use wherein said second metabolite is ethanol or glycerol or an undesirable aroma component naturally produced by a lactic acid bacterial cell.

Another preferred use of said microbial cell is in preparation of a drinkable or edible product or in a production of a first metabolite for use in said drinkable or edible product, said first metabolite having and/or providing a desirable organoleptic quality to said product. Preferably the first metabolite is ethanol or, when the microbial cell is a lactic acid bacterial cell, an aroma component produced by said lactic acid bacterial cell, preferably acetoin and/or diacetylactis.

A much preferred use of said microbial cell in said preparation of said drinkable or edible product is that of a microbial cell according to the invention, preferably a yeast cell or a lactic acid bacterial cell, further producing a second metabolite, said production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell, said decreased production resulting in a provision of a desirable organoleptic quality to

said product. There is also provided a use of a microbial cell, preferably a yeast cell or a lactic acid bacterial cell, in a preparation of a functional food.

In yet another aspect of the invention there is provided a method of producing a first metabolite, said method comprising the steps of

i) cultivating a microbial cell in a suitable growth medium and under such conditions that said microbial cell is producing a first metabolite

and optionally

ii) isolating said first metabolite in a suitable form,

and further optionally

iii) purifying said isolated first metabolite.

The method comprises cultivation of any microbial cell including a microbial eukaryotic cell or a microbial prokaryotic cell. Among microbial eukaryotes are many yeast and fungal cells preferred, such as yeast cells of the species *Saccharomyces*, *Schizosaccharomyces* and *Pichia*, such as e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and the like, as well as algae such as e.g. *Chlamydomonas reinhardi*, slime moulds such as e.g. *Dictyostelium discoideum* and filamentous fungi. Preferred filamentous fungi are species of *Neurospora* such as e.g. *Neurospora crassa*, and species of *Aspergillus* such as *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* species such as e.g. *Penicillium chrysogenum*, *Penicillium roqueforti* and *Penicillium camemberti*. Particularly preferred are industrially relevant yeast cells, slime moulds and filamentous fungi providing production of products such as e.g. antibiotics, steroids, pigments, enzymes, organic alcohols and acids, amino acids, polysaccharides and the like.

Among preferred microbial prokaryotes are bacterial cells such as Gram-positive species such as *Bacillus* species like e.g. *Bacillus subtilis*, *Bacillus thuringensis*, *Bacillus li-*

cheniformis, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus lentus* and *Bacilus stearothermophilus*, species of *Corynebacterium* like e.g. *Corynebacterium glutamicum*, and species of *Propionibacterium* as well as Gram-negative species such as *Escherichia coli*. Particularly preferred are also lactic acid bacterial species such as e.g. *Lactococcus lactis*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. diacetylactis*, *Leuconostoc* species such as e.g. *Leuconostoc oenos*, *Lactobacillus* species, such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus delbrückii subsp. bulgaricus*, and *Lactobacillus helveticus*, *Pediococcus* species, *Brevibacterium* species, *Propionibacterium* species and similar industrially relevant species like e.g. *Bifidobacterium*.

Embodiments of this aspect of the invention comprise a method wherein said first metabolite is either a primary metabolite or a secondary metabolite. Particularly preferred metabolites are selected from the group consisting of an alcohol, an acid, ethanol, glycerol, acetic acid, lactic acid, propionic acid, a β-lactam, a polyketide, a terpene, a steroid, a quinone, a coumarin, a flavonoid, an alkaloid, a piperidine, a pyridine, and the like. When the microbial cell is a lactic acid bacterium, said metabolite is preferably diacetyl, acetaldehyde, 2,3-butylene glycol, acetoin, or lactic acid.

Said metabolite may be produced by a cell further capable of producing a gene product heterologous to said cell, preferably a product selected from the group consisting of an a protease, an amylase, a cellulase, a β-glucanase, an endoglucanase, a phosphatase, a xylanase, a lipase, a β-lactamase, a β-galactosidase, a β-glucoronidase, and a xylosidase.

The method according to the invention pertains in one embodiment to an increased production of said first metabolite, such as a substantially increased production, as compared to the production of said first metabolite in a comparable wild-type cell or a comparable isolated microbial cell. Accordingly, there is provided a method by which said production of said first metabolite is increased at least by a factor of 1.01, such as 1.02, for example 1.03, such as a factor of at least 1.04, for example 1.05, such as 1.06, for example 1.07, such as 1.08, for example 1.09, such as 1.10, for example 1.11, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8,

such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example at least 100, such as 150, for example 200, such as 250, for example 300, such as 350, for example 400, such as 500, for example 600, such as 800, for example at least 1000, such as 1500, for example 2000, such as 2500, for example 3000, such as 3500, for example 4000, such as at least 5000, for example 6000, such as 8000, for example at least 10000, such as 15000, for example 20000, such as at least 25000, for example 30000, such as 35000, for example 40000, such as a factor of at least 50000.

When being isolated or when being isolated and purified, said metabolite is isolated or isolated and purified according to any available state of the art techniques for isolating or isolating and purifying a metabolite.

In one particularly preferred embodiment of the invention, the method pertains to the production in a yeast cell or in a lactic acid bacterial cell of a first metabolite such as a primary or secondary metabolite, preferably a primary metabolite and more preferably an alcohol or an acid, such as e.g. ethanol, glycerol, acetic acid and propionic acid, with ethanol being particularly preferred. Most preferred is a method wherein said cell is a yeast cell and the first metabolite is either ethanol or glycerol.

In another embodiment of the method according to the invention, there is provided a microbial cell, preferably a yeast cell or a lactic acid bacterial cell, said cell further producing a second metabolite, the production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell. In one embodiment, said decrease of said production of said second metabolite is at least 2 percent, such as 4 percent, for example at least 6 percent, such as 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, for example 18 percent, such as at least 20 percent, for example 24 percent, such as at least 30 percent, for example 35 percent, such as at least 40 percent, for example 50 percent, such as 60 percent, for example at least 70 percent, such as 80 percent, for example at least 90 percent, such as decreased

by at least 92 percent, for example 94 percent, such as 96 percent, for example 98 percent, such as decreased by 99 percent or decreased to such an extent that said second metabolite is virtually unassayable using state of the art assays for identifying and/or quantifying said second metabolite.

In one preferred embodiment the second metabolite is glycerol. In another preferred embodiment, when the cell is a lactic acid bacterial cell, the second metabolite is an undesirable aroma component naturally produced by a lactic acid bacterial cell.

There is also provided a method for generating an alternative re-oxidation of a reduced coenzyme, said method, in one embodiment, consisting essentially of providing in a microbial cell a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADH and/or NADPH re-oxidation for use in providing, supplementing and/or increasing a pool of intracellular NAD and/or NADP, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

Also, there is provided a method for generating an alternative reduction of an oxidised coenzyme, said method consisting essentially of providing in a microbial cell a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NAD and/or NADP reduction for the purpose of providing, supplementing and/or increasing a pool of intracellular NADH and/or NADPH, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In a further aspect there is provided a method of constructing a microbial cell, said method comprising introducing into said cell a capability and/or increasing a capability of said cell to express a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said expression of said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity. In a preferred embodiment, the first expressible enzyme activity is that of SEQ ID NO:2, or a

functional derivative thereof, as encoded by the nucleotide sequence designated SEQ ID NO:1, or a functional derivative thereof.

In a further aspect there is provided a method of constructing a microbial cell, said method comprising the steps of

i) operably linking a nucleotide sequence encoding a transhydrogenase activity with an expression signal not natively associated with said nucleotide sequence, and optionally

ii) operably linking a nucleotide sequence encoding a glutamate synthase activity with an expression signal not natively associated with said nucleotide sequence, and further optionally

iii) operably linking a nucleotide sequence encoding a glutamine synthetase activity with an expression signal not natively associated with said nucleotide sequence, and even further optionally

iv) reducing the expression of and/or eliminating a nucleotide sequence encoding a glutamate dehydrogenase activity in said cell, and

v) introducing said operably linked nucleotide sequence obtained under i), and optionally under ii) and iii), into a microbial cell, optionally the microbial cell obtained under iv), wherein said expression signal is expressible and, when expressed, results in expression of said operably linked nucleotide sequence.

In a further aspect there is provided a method of constructing a microbial cell, said method comprising introducing into said cell a capability and/or increasing a capability of said cell to express a first expressible enzyme activity which, when expressed in said microbial cell, is controlling an intracellular redox system of said cell, said expression of said first expressible enzyme activity in said microbial cell being operably linked to an expression signal not natively associated with said first expressible enzyme activity.

The term introducing into said cell a capability to express an expressible enzyme activity shall be understood to comprise any means by which said cell subsequently becomes capable of increasing an expression of said expressible enzyme activity. Accordingly, in one embodiment according to the method of the invention, said capability is a first nucleotide sequence comprising an expression signal, said expression signal being operably linked to a second nucleotide sequence, said second nucleotide sequence comprising said expressible enzyme activity. Said expression signal may direct a substantially constitutive expression, a constitutive expression during growth of said cell in a particular growth phase, an inducible expression in response to the presence and/or level of an inducer or the absence and/or level of a repressor.

The expression signal is preferably a regulable expression signal such as a regulable transcription initiation signal and/or a regulable translational initiation signal, such as an expression signal regulable in response to an alteration in a value, level and/or concentration of a factor such as a physiological growth parameter, preferably a parameter selected from the group consisting of pH, temperature, salt content including osmolarity, anaerobicity, aerobicity including oxygen level, energy level including a membrane potential and a proton motive force.

The promoter is preferably a promoter being either growth phase regulated, inducible and/or repressible and/or, in a natural host organism, directing expression of a gene encoding a gene product involved in mediating a reaction of a biosynthetic pathway and/or a major metabolic pathway, preferably a pathway selected from the group of pathways consisting of glycolysis, gluconeogenesis, citric acid cycle, and pentose phosphate pathway.

The expression signal may be further regulated by an upstream activating sequence (UAS), by an enhancer element or by a silencer element. The person skilled in the art will be aware of general molecular biology techniques for use in the construction in vitro of a recombinant DNA molecule. Such techniques are described e.g. in Sambrook et al. (1989) and in Old and Primrose (ibid.). Said skilled artisan will further be aware of the academic literature including general textbooks on molecular biology and genetic engi-

neering and he will be able to combine various expression signals such as putative or recognised promoter regions with a range of regulatory nucleotide sequences generally known to exert an effect on the level of gene expression. The skilled person is able to monitor gene expression by construction of suitable transcriptional and/or translational fusions of an expression signal to a reporter gene generally available in the art. An expression signal can be a cloned expression signal or an in vitro synthesised expression signal. Expression signals in prokaryotic microbial cells are known to comprise so-called –35 and –10 regions and numerous examples of such regions are available from various databases.

Expression signals may be optimised by increasing the promoter strength, by adjusting translational initiation sequences, by optimising the choice of codons by using so-called highly expressed codons, by adjusting the secondary structure of the mRNA, by increasing the efficiency of transcriptional termination, by increasing or decreasing a copy number of a vector, or by increasing or decreasing the stability of said vector.

The microbial cell is preferably a fungal cell, a yeast cell, or a bacterial cell. Particularly preferred is a microbial eukaryotic cell or a microbial prokaryotic cell. Among microbial eukaryotes are many yeast and fungal cells preferred, such as yeast cells of the species *Saccharomyces*, *Schizosaccharomyces* and *Pichia*, such as e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and the like, as well as algae such as e.g. *Chlamydomonas reinhardi*, slime moulds such as e.g. *Dictyostelium discoideum* and filamentous fungi. Preferred filamentous fungi are species of *Neurospora* such as e.g. *Neurospora crassa*, and species of *Aspergillus* such as *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* species such as e.g. *Penicillium chrysogenum*, *Penicillium roqueforti* and *Penicillium camemberti*. Particularly preferred are industrially relevant yeast cells, slime moulds and filamentous fungi providing production of products such as e.g. antibiotics, steroids, pigments, enzymes, organic alcohols and acids, amino acids, polysaccharides and the like.

Among preferred microbial prokaryotes are bacterial cells such as Gram-positive species such as *Bacillus* species like e.g. *Bacillus subtilis*, *Bacillus thuringensis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus lentus* and *Bacilus*

stearothermophilus, species of *Corynebacterium* like e.g. *Corynebacterium glutamicum*, and species of *Propionibacterium* as well as Gram-negative species such as *Escherichia coli*. Particularly preferred are also lactic acid bacterial species such as e.g. *Lactococcus lactis*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. diacetylactis*, *Leuconostoc* species such as e.g. *Leuconostoc oenos*, *Lactobacillus* species, such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus delbrückii subsp. bulgaricus*, and *Lactobacillus helveticus*, *Pediococcus* species, *Brevibacterium* species, *Propionibacterium* species and similar industrially relevant species like e.g. *Bifidobacterium*.

In one preferred embodiment according to the method of the invention, the first expressible enzyme activity is preferably any transhydrogenase activity and more preferably any pyridine nucleotide transhydrogenase activity, or any activity which, when expressed, results in an increased level of at least one intracellular coenzyme in its oxidised or reduced form, preferably a coenzyme in its oxidised/reduced form selected from the group consisting of FAD/$FADH_2$, NAD/NADH and NADP/NADPH. Said first expressible enzyme activity, when expressed, may also provide an increased level of at least one intracellular redox system consisting of a coenzyme in its corresponding oxidised and reduced forms, said redox system being preferably selected from the group consisting of FAD/$FADH_2$, NAD/NADH and NADP/NADPH.

In a preferred embodiment, said first expressible enzyme activity is that of the transhydrogenase activity encoded by *CTH* of *Azotobacter vinelandii* as harboured by *Saccharomyces cerevisiae* TN4 deposited under DSM Accession Number 12267, or a functionally equivalent activity within the meaning of that term as defined herein above. Said transhydrogenase activity in a preferred embodiment is operably linked to an expression signal not natively associated with said activity.

The method of the invention further comprises, in a particularly preferred embodiment, a further step of freezing or freeze-drying the microbial cell in the preparation of a reconstitutable lyophilisate.

In one presently preferred embodiment the microbial cell is a lactic acid bacteria as exemplified herein, and said first expressible enzyme activity is, at least in its native host organism, a cytoplasmic transhydrogenase, said expression of said cytoplasmic transhydrogenase resulting in an altered and/or novel product formation and/or metabolite production of said lactic acid bacteria.

An example of said novel product and/or metabolite formation is given herein below. Lactic acid bacteria metabolise pyruvate through a number of different pathways. The metabolite is converted into lactate by lactate dehydrogenase, into acetyl-CoA and CO_2 by pyruvate dehydrogenase, into formate by pyruvate formate lyase and into acetolactate and CO_2 by acetolactate decarboxylase. The carbon flux distribution through these pathways is dependent on the external growth conditions. This control is exerted through changes in the intracellular NADH/NAD^+ ratio (C. Garrigues, P. Loubiere, N. D. Lindley and M. Cocaign-bousquet (1997). *J. Bacteriology* **179**, 5282-5287; F. Lopez de Felipe, M. Kleerebezem, W. M. de Vos and J. Hugenholtz (1998). *J. Bacteriology* **180**, 3804-3808). This is illustrated by the observations from physiological studies of lactic acid bacteria that are listed below.

Lactic acid bacteria with an increased formation of the secondary metabolite diacetyl are relevant for a number of industrial applications. It has been shown that overexpression of NADH-oxidase from *Streptococcus mutans* in *L. lactis* results in a shift from homolactic (production of lactic acid) to mixed acid fermentation (production of lactic acid, acetic acid, acetoin and diacetyl) under aerobic growth conditions. This effect is ascribed to a decrease in the intracellular NADH/NAD^+ ratio of the recombinant strain (F. Lopez de Felipe, M. Kleerebezem, W. M. de Vos and J. Hugenholtz (1998). *J. Bacteriology* **180**, 3804-3808). Expression of the cytoplasmic transhydrogenase in lactic acid bacteria is expected to have a similar effect on the NADH/NAD^+ ratio if the reaction occurs in the direction from NADH to NADPH.

It has been shown that the product formation by *L. lactis* changes when the carbon source is shifted from glucose to lactose and that this effect is due to a lower flux through glycolysis, resulting in a lower NADH/NAD^+ ratio (C. Garrigues, P. Loubiere, N. D. Lindley and M. Cocaign-bousquet (1997). *J. Bacteriology* **179**, 5282-5287). Thus, under an-

aerobic growth conditions on glucose the majority (93%) of the carbon source was converted into lactate, while only 4% of the carbon source was converted into lactate when lactose was used as carbon source. The remaining part was converted into formate, acetate and ethanol. The authors state that lactate formation is reduced due to a 3-fold lower NADH/NAD^+ ratio during growth on lactose as compared with glucose, resulting in deactivation of lactate dehydrogenase. Instead, the carbon flux towards the pyruvate node is redirected towards formation of acetate, ethanol and formate in order to synthesise ATP and reoxidise NADH. Expression of the cytoplasmic transhydrogenase in lactic acid bacteria is expected to have a similar effect on the NADH/NAD^+ ratio if the reaction occurs in the direction from NADH to NADPH. Thus, it is expected that cultivation of a transhydrogenase-containing recombinant strain of lactic acid bacteria under anaerobic growth conditins will result in production of several new byproducts besides lactate.

Since lactate dehydrogenase is activated by a high NADH/NAD^+ ratio it is expected that the flux towards lactate can be increased by expressing transhydrogenase in lactic acid bacteria under conditions where the transhydrogenase reaction occurs in the direction from NADPH to NADH.

The invention will be further exemplified in the below provided examples directed to preferred embodiments of the invention. It will be understood that the invention is by no means limited to said examples. The examples include figures illustrating the invention and the legends to said figures are listed below.

FIGURE LEGENDS

Figure 1 shows a comparison of the amino acid sequences of *CTH* of *Azotobacter vinelandii* as described herein and a pyridine nucleotide transhydrogenase from *P. fluorescens* (French *et al.*, 1997).

Figure 2 shows the consumption of glucose and formation of ethanol, glycerol and carbon dioxide in strain TN4.

Figure 3 shows the consumption of glucose and formation of ethanol, glycerol and carbon dioxide in strain TN3.

Figure 4 shows the formation of succinate, pyruvate, acetate and 2-oxoglutarate in strain TN4.

Figure 5 shows the formation of succinate, pyruvate, acetate and 2-oxoglutarate in strain TN3.

EXAMPLES

EXAMPLE 1

Expression of a transhydrogenase activity in *Saccharomyces cerevisiae*

Introduction

This example contains a description of a cloning of a gene encoding a cytoplasmic transhydrogenase from *A. vinelandii* and the expression of said gene in a *S. cerevisiae* strain derived from the industrial model strain *S. cerevisiae* CBS8066 (Nissen *et al.*, 1997; Verduyn *et al.*, 1990). The constructed strain has been cultivated anaerobically in batch culture in a high performance bioreactor in order to perform a quantitative analysis of the effect of transhydrogenase expression on product formation and on the intracellular concentrations of NADH, NAD^+, NADPH and $NADP^+$.

Materials and Methods

Microorganisms and their maintenance. All *Saccharomyces cerevisiae* strains were generated from *Saccharomyces cerevisiae* T23D which was kindly provided by Jack Pronk from the Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, The Netherlands. The strain was maintained at 4°C on YPG agar plates, monthly prepared from a lyophilized stock kept at -80°C.

Escherichia coli DH5α (F⁻ F80d*lacZ* DM15 D(*lacZYA- argF*) U169 *deoR recA1 endA1 hsdR17*(r_k^- m_k^-) *supE44* l⁻ *thi-1 gyra96 relA1*) (GIBCO BRL, Gaithersburg, MD. USA) was used for subcloning. *Azotobacter vinelandii* ATCC 478 was purchased from Centraalbureau voor Schimmelcultures (Baarn & Delft, The Netherlands). The strain was maintained at 4°C on agar plates consisting of Winegradskis media monthly prepared from a lyophilized stock kept at -80°C.

Preparation of DNA. Plasmid DNA from *E. coli* was prepared with Qiagen colums (Qiagen GmbH, Dûsseldorf, Germany) following the manufacturer's instructions. For the purification of DNA fragments used for cloning experiments, the desired fragments were separated on 0.8% agarose gels, excised and recovered from agarose using the Qiagen DNA isolation kit (Qiagen GmbH, Dûsseldorf, Germany). Chromosomal DNA from *Saccharomyces* cerevisiae and Azotobacter *vinelandii* was extracted as follows. Cells were grown in Winegradskis media in shake flasks and harvested at OD=1.5. 10 mg of wet cells were resuspended in 0.5 ml Tris-Cl (pH 8.0) and quenched with 0.5 ml glass beads (size 250-500 microns) in the presence of 0.5 ml Tris-saturated phenol (pH 8.0). The DNA was extracted from the phenol phase with chloroform, precipitated with 98% ethanol and resuspended in TE buffer. RNA in the extract was removed by treatment with RNAaseA (purchased from Promega) and finally the DNA was purified by precipitation with ethanol/lithium chloride and resuspended in TE buffer. The DNA primers were purchased from DNA Technology (Aarhus, Denmark).

Plasmid constructions. A plasmid, pRY253, containing the DNA sequence from 1100 bp upstream of *HO* to 1500 bp downstream of the open reading frame was digested with the restriction enzyme SspI and two fragments of 594 bp (-119 bp to 475 bp) and 180 bp (+50 bp to 230 bp) was isolated. The fragments were ligated into the SmaI and EcoRV sites of plasmid pFA6A-kanMX3 (Wach *et al.*, 1994), respectively. The correct direction of the inserts was verified by PCR. The resulting plasmid, pHOdel, was digested with BamHI and SpeI prior to transformation and a 3350 bp fragment consisting of *G418*^{r} flanked by the two inserts was isolated.

A plasmid, pRB58, containing the chromosomal region around *SUC2* was kindly donated by Danisco Biotechnology (Copenhagen, Denmark). A 3.9-kb fragment (-906 bp to +1383) containing the open reading frame and its promoter and terminator was isolated by digestion with EcoRI and ClaI. The EcoRI site was made blunt by treatment with

Klenow fragment and the fragment was inserted into the StuI/BstBI sites of the *URA3* gene in the yeast integration plasmid YIp5. The resulting construct, pSUC2, was linearised with PstI prior to transformation.

All restriction enzymes were purchased from Promega and New England Biolabs and used as recommended by the manufacturer.

Transformation of *E. coli* and *S. cerevisiae*. *E. coli* DH5α was transformed by electro-transformation using the Bio-Rad electroporation equipment (Biorad Laboratories, Richmond, USA). Transformants were selected on L broth plates containing 100 mg/ml ampicillin. *S. cerevisiae* cells were made competent for plasmid uptake by treatment with lithium acetate and polyethyleneglycol (Schiestl & Gietz, 1989). 5 µg of DNA was used for each transformation. Transformants were plated directly on selective media except for the G418 resistant transformants. These were suspended in YPD for 24 hours prior to plating on selective media in order to obtain expression of the G418 resistance gene. Correct integration of the fragments from pHOdel and pSUC2into the chromosome was verified by PCR analysis using extracted DNA from the transformants.

Generation of a haploid strain with uracil auxotrophy from *S. cerevisiae* T23D. The diploid *Saccharomyces cerevisiae* T23D (Wenzel *et al.*, 1992) is a meiotic progeny of the tetraploid, industrial model strain CBS8066. The strain was chosen as the parent strain in this study in order to facilitate the introduction of genetic changes in the organism while maintaining the genetic background of CBS8066. In order to obtain a stable, haploid strain, which was isogenic to T23D, *HO* was deleted in one of the alleles in T23D. The gene encodes a homothallic switching endonuclease that enables meiotic progeny of a diploid to switch mating type. T23D was transformed with a 3350 bp fragment from pHOdel and transformants with integration of the fragment in the *HO* locus were selected on YPD plates containing 300 mg/l G418. Loop out of *G418*^{r} by homologue recombination of the two direct repeats in the insert (Wach *et al.*, 1994) was obtained with a frequency of 1/10000 colonies after cultivation of one of the transformants in non-selective YPD media for 30 generations. It was verified by PCR that the loop out transformants contained both the wildtype *HO* and the expected deletion in the gene (results not shown). One of the loop out transformants was sporulated and the resulting spores were tested for mating types.

Approximately 50 % of the tested colonies were stable haploids with either an a- or α-mating type, while the rest were diploids. One of the haploid strains with α mating type was designated TN1 and used in the genetic manipulations described below.

In order to obtain a strain with auxotrophy towards uracil, a 3.9-kb fragment containing *SUC2*, encoding invertase, was inserted into the *URA3* locus of TN1. This was done by transformation with pSUC2 after linearisation with PstI. Transformants were selected on minimal media containing sucrose and uracil, since T23D and its haploid progeny did not grow on this carbon source while the transformants did. Single colonies of the transformants were isolated on minimal media containing sucrose and 5-fluoroorotic acid. The latter is lethal to cells with an intact *URA3* locus. The transformants did not grow in the absence of uracil in the media confirming their auxotrophy towards this compound. One of the uracil auxotrophs was designated TN2 and used for expression of the cytoplasmic transhydrogenase as described below.

Expression of *CTH* in *S. cerevisiae*. A multi copy plasmid, Yep24-pPGK, constructed from Yep24, containing the promoter and terminator of *PGK* was provided by Mikael Anderlund (Walfridsson *et al.*, 1997). *CTH* was ligated into YEp24 behind the strong constitutive promoter of *PGK* resulting in plasmid Yep24-pPGK-CTH. This plasmid was transferred into strain TN2 resulting in strain TN4. Yep24-pPGK was transferred into strain TN2 resulting in strain TN3. This strain served as a negative control in the physiological studies.

Medium in the batch cultivations. The yeast was cultivated in a mineral medium prepared according to Verduyn *et al.*(1990). Vitamins were added by sterile filtration following heat sterilization of the medium. The concentrations of glucose and $(NH_4)_2SO_4$ was 25 g l^{-1} and 7.5 g l^{-1}, respectively. Growth of *S. cerevisiae* under anaerobic conditions requires the supplementary addition to the medium of ergosterol and unsaturated fatty acids, typically in the form of Tween 80 (Andreasen & Stier, 1953; Libudzisz *et al.*, 1986). Ergosterol and Tween 80 were dissolved in 96 %(v/v) ethanol and the solution was autoclaved at 121°C for 5 min. The final concentrations of ergosterol and Tween 80 in the medium were 4.2 mg g DW^{-1} and 175 mg g DW^{-1}, respectively. To prevent foaming 75 µl l^{-1} antifoam (Sigma A-5551) was added to the medium.

Experimental setup for the batch cultivations. Anaerobic batch cultivations were performed at 30°C and at a stirring speed of 800 rpm in in-house manufactured bioreactors. The working volume of the reactors were 4.5 liters. pH was kept constant at 5.00 by addition of 2 M KOH. The bioreactors were equipped with off-gas condensers cooled to 2°C. The bioreactors were continuously sparged with N_2 containing less than 5 ppm O_2, obtained by passing N_2 of a technical quality (AGA 3.8), containing less than 100 ppm O_2, through a column (250x30 mm) filled with copper flakes and heated to 400°C. The column was regenerated daily by sparging it with H_2 (AGA 3.6). A mass flow controller (Bronkhorst HiTec F201C) was used to keep the gas flow into the bioreactors constant at 0.50 l nitrogen min^{-1} $liter^{-1}$ Norprene tubing (Cole-Parmer Instruments) was used throughout in order to minimize diffusion of oxygen into the bioreactors. The bioreactors were inoculated to an initial biomass concentration of 1 mg l^{-1} with precultures grown in unbaffled shake flasks at 30°C and 100 rpm for 24 hours.

The anaerobic batch cultivations of strains TN1, TN3 and TN4 were each carried out three times with identical results.

Determination of dry weight. Dry weight was determined gravimetrically using nitrocellulose filters (pore size 0.45 µm; Gelman Sciences). The filters were predried in a microwave oven (Moulinex FM B 935Q) for 10 min.. A known volume of culture liquid was filtered and the filter was washed with an equal volume of demineralized water followed by drying in a microwave oven for 15 min.. The relative standard deviation (RSD) of the determinations was less than 1.5 % based on triple determinations (n=3).

Analysis of medium compounds. Cell-free samples were withdrawn directly from the bioreactor through a capillary connected to a 0.45 µm filter. Samples were subsequently stored at -40°C. Glucose, ethanol, glycerol, acetic acid, pyruvic acid, succinic acid and 2-oxoglutarate were determined by HPLC using an HPX-87H Aminex ion exclusion column (RSD < 0.6 %, n=3). The column was eluted at 60°C with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Pyruvic acid, acetic acid and 2-oxoglutarate were determined with a Waters 486 UV meter at 210 nm whereas the other compounds were determined with a Waters 410 refractive index detector. The two detectors were connected in series with the UV detector first. The CO_2 concentration in the off-gas was determined using a Brüel & Kjær 1308

acoustic gas analyser (RSD = 0.02 %) (Christensen *et al.*, 1995). In a separate experiment the off gas from the bioreactor was bubbled through liquid nitrogen and the ethanol concentration in the frozen mixture of water, ethanol and acetaldehyde was determined by HPLC after evaporation of the N_2. Hereby the loss of ethanol through the reflux condenser of the bioreactor was determined to be between 4% and 9% of the ethanol formed by the bioreaction depending on the dilution rate (Schulze, 1995). In the carbon balances the measured ethanol fluxes were corrected for this loss through evaporation.

2-oxoglutarate was identified in the extracellular samples by an enzymatic assay. 100 µl ammonium (50mM), 40 µl NADPH (40 mM), 100 units of glutamate dehydrogenase (Boehringer Mannheim) and 1 ml KPO_4-buffer (50 mM, pH 7.0) was pipetted into a quartz cuvette. At time zero 100 µl of a sample was added and the absorbency at 340 nm was monitored.

Measurement of enzyme activities. Culture liquid was withdrawn from the bioreactor into an ice cooled beaker, centrifuged and washed twice with 50 mM Tris-Cl buffer (pH 7.5, 2°C). Subsequently the cells were resuspended in 5 ml 50 mM Tris-Cl buffer (pH 7.5, 2°C) whereafter they were distributed into precooled 2 ml eppendorf tubes containing 0.75 ml glass beads (size 250-500 microns). The cells were disrupted in a bead mill for 20 min. (0°C). The test tubes were centrifuged (20000 rpm, 20 min., 0°C) whereafter the supernatants were pooled in one test tube. During the following analyses the extract was kept on ice. Transhydrogenase activity was assayed as described by Voordouw *et al.* (1979). The molar extinction coefficient of tNADH and NAD(P)H was taken as 11300 liters mol^{-1} cm^{-1} (Cohen & Kaplan, 1970) and 6220 liter mol^{-1} cm^{-1}. The activities of hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were assayed as described by Postma *et al.* (1989).

Preparation of extracts for the measurements of intracellular nucleotides. The intracellular nucleotides were extracted from cells of *S. cerevisiae*, growing anaerobically in batch cultivations. 5.0 ml of culture liquid was withdrawn from the bioreactor and sprayed into 20 ml of 60% methanol (-40^{0}C) within 1 second. Except for the following changes, the further proceedings were carried out as described earlier for the cold methanol extraction method (de Koning & van Dam, 1992). Instead of storing the samples in a

freezer after quenching the cells in cold methanol, the nucleotides were extracted from the cells and quantified in one step to avoid degradation. Instead of using a neutral 2 mM Pipes buffer for collection of the nucleotides during the extraction, a 50 mM KPO_4-buffer (pH 5.0) was used in extraction of NAD^+ and $NADP^+$ while 50 mM Tris-Cl (pH 9.0) was used in extraction of NADH and NADPH. This was done to increase the stability of the compounds since the oxidised nucleotides degrade slower under acidic conditions while the reduced nucleotides degrade slower under alkalic conditions. The concentration of the nucleotides was quantified immediately after reducing the volume of the samples by evaporation under vacuum.

Determination of intracellular nucleotides. The content of NAD^+, NADH, $NADP^+$ and NADPH in the samples from the cold methanol extraction was determined enzymatically as described by Bergmeyer (1985). The nucleotide concentrations were quantified using standard curves for each compound.

Results

Purification of the cytoplasmic transhydrogenase from *A. vinelandii.* *Azotobacter vinelandii* contains a transhydrogenase located in the cytoplasm. A method for purification of the enzyme is described in the literature, but the structural gene has not been cloned (Voordouw *et al.*, 1980). The method was modified in this study and will be described in some detail below. *A.vinelandii* ATCC 478 was cultured in 14 litres of Winegradskis nitrogen-free medium at 37°C for 5 days and harvested by centrifugation (3000xg, 20 minutes) and resuspended in buffer A (50 mM Tris-Cl (pH 7.5) with 5 mM dithiothreitol). Phenylmethylsulfonyl fluoride was added to buffer A at a concentration of 1 mM and intracellular protein was extracted from 30 g cells (wet weight) by treating the cells with lysozyme followed by passage through a French press at approximately 14 MPa. These procedures were carried out at 2°C. Cell-free extract was isolated by ultracentrifugation (60000xg, 30 min, 2°C). The extract contained 710 units of transhydrogenase activity at a specific activity of 0.39 U/mg protein. The extract was loaded onto a 2',5'-ADP-Sepharose-4B affinity column with a 10-ml bed volume (1.2 x 9 cm, Pharmacia) at a flow rate of 10 ml/hour. The ligands of the column bind specifically to $NADP^+$-dependent dehydrogenases. After washing the column (20 bed volumes, 50 mM Tris-Cl

(pH 7.5), 5 mM $MgCl_2$, 5 µM FAD, 0.02 (w/v) NaN_3) at the same flow rate the attached proteins were eluted with washing buffer containing 4 mg 2'-AMP per ml. The eluate contained 398 units of transhydrogenase activity at a specific activity of 121 U/mg protein. The eluate was loaded onto a Sephadex G-75 gel filtration column (Pharmacia) with a 340-ml bed volume and fractions of 3 ml was collected. The protein content of the fractions was measured on-line with a spectrophotometer at OD280. The fractions containing transhydrogenase were identified by measuring the enzyme activity. Active fractions were pooled and concentrated by ultrafiltration with an Amicon ultrafiltration cell fitted with a membrane with a nominal M_r cut-off of 30000. The concentrate contained 287 units of transhydrogenase activity at a specific activity of 240 U/mg protein, corresponding to a 615-fold purification at a yield of 40%.

A fraction of the concentrate was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It showed two protein bands with the apparent M_r of approximately 38000 and 54000, respectively. Both protein bands were isolated from the gel for further analysis. The N-terminal sequence of the small SDS-PAGE protein band was determined by automated Edman degradation and was found to be M-K-V-Y-Y-D-K-D-A-D-L-S-I-I-Q-S-K-K-V-A-I. A similarity search through all databases available on the world wide web using the BLASTP 1.4.11 program (Altschul *et al.*, 1990) showed 76% identity and 100% similarity to the $NADP^+$-specific acetohydroxy acid isomeroreductase from *Rhizobium meliloti*, which has a M_r of 36518. The cells used in this work were grown in minimal medium while the growth medium used in the earlier purification of transhydrogenase is unknown. Since the enzyme is involved in *de novo* synthesis of branched chain amino acids, absence of the protein in the earlier purification procedure could be explained by the use of a rich growth medium.

Cloning of the structural gene encoding the cytoplasmic transhydrogenase from *A. vinelandii*. In the literature it is described that transhydrogenase from *A.vinelandii* has an apparent M_r of 54000 (Voordouw *et al.*, 1980). Since the isolated protein described above with this size was blocked in the N-terminus, it was digested with trypsin and the resulting polypeptides were separated by hydrofobicity on HPLC (reference). 5 polypeptides were sequenced by automated Edman degradation (underlined in Figure 1).

These were used to construct degenerate oligonucleotides that were used in all combinations as primers in PCR reactions with chromosomal DNA from *A. vinelandii* as template. In the reaction including the two primers 5'- G-T-(A/T/C/G)-T-A-(C/T)-A-A-(C/T)-T-A-(C/T)-G-A-(C/T)-G-T-(A/T/C/G)-G-T-(A/T/C/G)-G-T-(A/T/C/G)-A-T-(A/C/T) - 3' (SEQ ID NO:3) encoding residues 3 to 11 in Figure 1, and 5'- (A/G)-T-A-(A/G)-T-T-(A/G)-A-A-(A/T/C/G)-G-T-(A/T/C/G)-G-T-(A/G)-T-T-(A/T/G)-A-T-(A/G)-A-A-(A/G)-T-A- 3' (SEQ ID NO:4) encoding residues 437 to 445 in Figure 1, a DNA fragment of 1300 bp was obtained. This fragment was subcloned into the SmaI site of pUC18 and partly sequenced. The remaining 100 bp of the gene was identified by inverse PCR (Triglia *et al.*, 1988). Chromosomal DNA from *A. vinelandii* was digested with PvuII (Promega). No site for this restriction enzyme was present in the 1300 bp fragment while two sites on each side of the fragment turned out to be conveniently located for the further proceedings. Selfligation of the linearised chromosomal DNA fragments was obtained by treatment with T4 DNA ligase (Promega). From the partly known sequence of the 1300 bp fragment it was possible to construct two inverse primers (5'- A-C-T-G-C-T-T-C-G-G-C-T-A-T-C-A-G-G-C-T-T- 3' (SEQ ID NO:5) encoding the complementary strand to base pairs 34 to 54 and 5'-G-G-C-G-A-G-G-C-G-A-A-T-A-C-C-C-T-C-A-A-G- 3' (SEQ ID NO:6) encoding base pairs 1288 to 1308) that elongated in the direction away from the open reading frame. These were used in inverse PCR with the circular, chromosomal DNA as template resulting in the isolation of a 1300 bp inverse PCR fragment. Only the circular DNA preparations, originating from the digestion with PvuII, resulted in amplification of a well-defined DNA band. The fragment was subcloned into the SmaI site of pUC18. Sequencing of the inverse PCR fragment resulted in identification of a start and a stop codon with the expected distance to the known sequence of the original 1300 bp PCR fragment. Two primers with synthetic recognition sites for the restriction enzyme BglII and located at the start and stop codon of the open reading frame, respectively, was constructed (start: 5'- G-C-G-C-G-A-G-A-T-C-T-T-C-T-A-G-A-**A-T-G**-G-C-T-G-T-A-T-A-T-A-A-C-T-A-C-G-A-T- 3' (SEQ ID NO:7); stop: 5'- C-G-C-G-C-A-G-A-T-C-T-C-C-G-C-G-G-A-G-C-C-T-C-T-C-A-A-A-A-A-A-G-C-C-G-A-T- 3' (SEQ ID NO:8)). These were used to obtain the entire open reading frame of the structural gene by PCR, using chromosomal DNA from *A. vinelandii* as template. The isolated 1430 bp DNA fragment was subcloned into the SmaI site of pUC18. Two identical fragments obtained in independent PCR reactions were sequenced. The nucleotide sequence is listed in SEQ ID NO:1. It has approx. 83% identity to the published sequence of the structural gene encoding transhydro-

genase from *Pseudomonas fluorescens* (French *et al.*, 1997). The new sequence was designated *CTH* for Cytoplasmic Transhydrogenase.

Analysis of the amino acid sequence. The deduced amino acid sequence of CTHp is listed in SEQ ID NO:2. The protein has a M_r of 51345, as determined by the Compute pI/Mw program on the world wide web (Bjellqvist *et al.*, 1993). This is slightly lower than the M_r of 54000 expected from the migration position in SDS-PAGE. Furthermore, the amino acid composition fitted well with earlier determinations (Voordouw *et al.*, (1980); Middleditch *et al.*, 1972). A similarity search through all databases available on the world wide web using the BLASTP 1.4.11 program (Altschul *et al.*, 1990) showed 84% identity and 91% similarity to the protein sequence of the soluble pyridine nucleotide transhydrogenase from *P. fluorescens* (French *et al.*, 1997). As observed for the *P. fluorescens* protein, CTHp had a similarity of up to 52% to the pyridine nucleotide-disulphide oxidoreductases, *e.g.* dihydrolipoamide dehydrogenases, glutathione reductases and mercuric ion reductases. These are FAD flavoproteins which contain an active site with the consensus pattern G-G-X-C-(L/I/V/A)-X-G-C-(L/I/V/M)-P where the two cysteines are involved in the transfer of reducing equivalents from the FAD cofactor to the substrate (Kuriyan *et al.*, 1991). A similar pattern is observed in the two soluble transhydrogenases from amino acid 41 to 51 in Figure 1, but one of the cysteine residues is missing. Hence, the characteristic active site, that is observed in other flavoproteins, is absent in the soluble transhydrogenases. A high similarity of 75% was observed to an unknown dehydrogenase from *E. coli*. The protein is encoded by *udhA*, which is located next to *oxyR* in the chromosome of the bacterium. Since *oxyR* is a part of the positive regulation of genes involved in the defence against oxidative damage (Tao *et al.*, 1989), *udhA* could have a role in this process. Defence against oxidative stress is dependent on consumption of NADPH through glutathione reductase in most organisms and the high similarity between the two soluble transhydrogenases and *udhA* could indicate a physiological role of the three enzymes in synthesis of NADPH when the cell is subjected to oxidative stress. Two βαβ or Rossman fold motifs (G-X-G-X-X-G) forming the FAD and NAD(P) binding sites are observed at identical locations in both soluble transhydrogenases. This fits well with earlier findings where two different binding sites for nicotinamide nucleotides were identified in the soluble transhydrogenase of *P. aeruginosa* (Höjeberg *et al.*, 1976) and is also supported by a proposed model of the allosteric regulation of soluble transhydrogenases (Rydström *et al.*, 1987). One site binds both NADP(H) and NAD(H) and

constitutes the active site where transfer of reducing equivalents between the two cofactor systems occurs through a simple ping-pong mechanism (Widmer & Kaplan, 1977). The second site binds only to NADP(H) and is involved in allosteric regulation of the enzyme activity by NADP(H) and 2'-nucleotides. By sequence homology studies of several NAD^+-binding enzymes it has been found that the NAD(H) binding site of these enzymes in addition to the Rossman fold motif consists of an acidic residue (aspartate or glutamate) 18-19 residues upstream of the last glycine residue in the Rossman fold and a glycine residue 13 residues further upstream of the acidic residue (Olausson *et al.*, 1995). This is observed for the Rossman motif located from amino acid 12 to 17 which indicates that NAD(H) binds to this site and thus, that it is the active site of the enzyme.

Anaerobic batch cultivations. Anaerobic, glucose-limited batch cultivations of strains TN1, TN3 and TN4 were carried out in order to analyse the effect of *CTH* expression on the maximum specific growth rate, product formation, enzyme activities and the intracellular levels of the four nucleotides NAD, NADH, $NADP^+$ and NADPH. Figures 2 and 3 show the consumption of glucose and formation of ethanol, glycerol and carbon dioxide in strains TN4 and TN3, respectively.

In order to verify correct expression of *CTH* in strain TN4, cells of strains TN1, TN3 and TN4 were sampled from the batch cultivations at different time points in the exponential growth phase and disrupted as described above. The specific enzyme activities of transhydrogenase, glucose-6-phosphate dehydrogenase (G6PDH) and hexokinase/glucokinase (HXK+GLK) in the extracts were measured and did not vary throughout the exponential growth phase (Table 1).

TABLE 1

Strain	CTHp activity	HXK+GLK activity	G6PDH Activity	G6PDH/ (HXK+GLK)
TN1	0	3.15 ± 0.29	0.61 ± 0.04	0.19 ± 0.02
TN3	0	3.18 ± 0.21	0.63 ±0.05	0.20 ± 0.02
TN4	4.53 ± 0.16	3.10 ± 0.32	0.62 ±0.03	0.20 ± 0.02

Table 1 *Specific enzyme activities measured in vitro in cell free extracts from samples from the exponential growth phase of strains TN1, TN3 and TN4 grown in anaerobic, glucose-limited batch cultivations.*

No transhydrogenase activity could be detected in extracts from strains TN1 and TN3 while a specific transhydrogenase activity of 4.53 U/mg protein was measured in the extract from strain TN4 containing plasmid Yep24-pPGK-CTH. Hence, it was concluded that expression of *CTH* in *S. cerevisiae* was successful. Based on the specific activity of 575 U/mg for the purified transhydrogenase from *A. vinelandii* (Voordouw *et al.*, 1980), it was estimated that CTHp formed approximately 0.8% of the protein pool in strain TN4. This level is approximately 10 times higher than in the protein extracts from *A. vinelandii* from this study. As described earlier an important role of the pentose phosphate pathway (PPP) is to reduce $NADP^+$ to NADPH and thus, it could be expected that expression of transhydrogenase would have an effect on the activity of this pathway. The specific activities of G6PDH and HXK+GLK were measured to detect differences in the carbon flux through the PPP in the three strains. It has been shown that the ratio between the specific activities of G6PDH and HXK+GLK is a reasonably good estimate of the ratio between the flux through PPP and the glycolytic flux (Nissen *et al.*, 1997). No difference could be detected in the specific activities of the enzymes in extracts from TN1, TN3 and TN4. This indicated that expression of the soluble transhydrogenase in strain TN4 had no effect on the flux through the pentose phosphate pathway.

Formation of a new product was observed in the cultivations of TN4 (Figure 4) as compared to cultivations of TN3 (Figure 5). The retention time of this product in the ion exclusion column indicated that it could be 2-oxoglutarate. An enzymatic assay was set up in order to determine if 2-oxoglutarate was present in samples from the batch cultivations of TN1, TN3 and TN4 (described in materials and methods). The assay was not optimised for a quantitative determination of the 2-oxoglutarate content in the samples, but it clearly demonstrated the presence of the compound in samples from the cultivations of TN4 while no 2-oxoglutarate could be detected in samples from the cultivations of TN1 and TN3. Quantification of 2-oxoglutarate formation was done by HPLC. Small amounts of the compound was found in extracellular metabolite samples from the cultivations of strain TN1 and TN3 while 4.5% of the carbon source was converted into 2-oxoglutarate in the cultivations of TN4 (Table 2).

TABLE 2

Product	TN1	TN3	TN4
Ethanol	0.494	0.493	0.451
Glycerol	0.091	0.093	0.118
CO_2	0.275	0.271	0.262
Succinate	0.005	0.005	0.006
Pyruvate	0.005	0.005	0.001
Acetate	0.005	0.005	0.007
Biomass	0.111	0.112	0.102
2-oxoglutarate	0.002	0.007	0.045
Total	0.988	0.991	0.992

Table 2 *Product yields in anaerobic, glucose limited cultivations. Unit: c-moles per c-mole glucose.*

During growth with ammonium as nitrogen source, a central step in assimilation of this compound is catalysed by glutamate dehydrogenase. Here, 2-oxoglutarate and ammonia is converted into glutamate and NADPH is oxidised to $NADP^+$ (Moye *et al.*, 1985). Secretion of 2-oxoglutarate from cells containing the cytoplasmic transhydrogenase strongly indicates that the flux through this reaction is to slow to consume the amount of 2-oxoglutarate that is synthesised in the TCA cycle. In an earlier study, a metabolic flux analysis of continuous cultivations of *S. cerevisiae* CBS8066 showed that, independent of the dilution rate, 51% of the cytoplasmic NADPH was consumed in the reaction catalysed by glutamate dehydrogenase (Nissen *et al.*, 1997). If NADPH and NAD^+ are converted into NADH and $NADP^+$ by the cytoplasmic transhydrogenase, the intracellular pool of NADPH could decrease significantly, resulting in a reduction of the conversion rate through reactions that use NADPH as cofactor. Hence, 2-oxoglutarate is probably secreted by the cell due to consumption of NADPH by transhydrogenase.

In contrast to the cultivations of TN1 and TN3, pyruvate was consumed at the end of the cultivations of TN4 (Figure 4). The nitrogen flow through the batch reactors was controlled at 0.5 l/l/min resulting in strictly anaerobic conditions. Hence, the presence of small amounts of oxygen in the reactor could not explain the consumption of pyruvate. As described above, transhydrogenase probably converts NADPH to NADH in the cytoplasm of strain TN4. In the compartment pyruvate can be converted into acetaldehyde and further into acetate by pyruvate decarboxylase and the $NADP^+$-dependent cytoplasmic aldehyde dehydrogenase, respectively (Tamaki & Hama, 1982; Meaden *et al.*, 1997; Nissen *et al.*, 1997). Hereby, 1 mole of NADPH is synthesised in the cytoplasm per mole of pyruvate consumed. As can be observed in Figure 4, acetate is formed when pyruvate is metabolised. 0.38 mmoles/l of pyruvate was consumed from t=2393 minutes to t=2635 minutes and in the same period exactly 0.38 mmoles of acetate was formed. This strongly indicates that pyruvate is converted to acetate at the end of the cultivations of strain TN4 to form NADPH in the cytoplasm.

The maximum specific growth rates, μ_{mas}, of TN1 and TN3 were 0.39 h^{-1} and 0.35 h^{-1}, respectively. The reduction in the maximum specific growth rate of TN3 must be due to the presence of the control plasmid in multiple copies. Introduction of plasmid Yep24 pPGK-CTH into strain TN4 resulted in a further decrease in μ_{mas} to 0.18 h^{-1}. In the cultivations a minimal medium was used whereby the intracellular precursors for biomass synthesis had to be synthesised by the cells from glucose and ammonia. In this process glutamate is involved in several reactions as a donor of ammonium in the formation of proteins, RNA and DNA (Jones & Fink, 1982). As described above the rate of conversion of 2-oxoglutarate towards glutamate probably was significantly reduced in TN4 due to a lower level of NADPH in this strain. The reduced maximum specific growth rate of TN4 could be due to this decrease in the synthesis rate of glutamate.

From Table 2 it is seen that the measured products could account for about 99% of the consumed glucose. Also a balance of the degree of reduction of substrate and products closed within 3%. The degree of reduction of the missing carbon was 6.5, indicating that formation of ethanol was measured too low. Ethanol formation in TN4 was 11% lower than in the control strains. This could be explained by a reduction in consumption of ATP in biomass synthesis since the biomass yield of TN4 also decreased compared to TN1

and TN3, by 13 %. During anaerobic growth of *S. cerevisiae* ATP is only formed through synthesis of ethanol. An increase in the glycerol yield from 0.093 c-mole/c-mole glucose in strains TN1 and TN3 to 0.117 c-mole/c-mole glucose in strain TN4 was observed. Even though the biomass yield was lower in strain TN4, the large increase in formation of 2-oxoglutarate in this strain as compared to TN1 and TN3 resulted in a significant increase in the surplus formation of NADH. As described earlier glycerol is formed during anaerobic growth of *S. cerevisiae* in order to reoxidise NADH formed in synthesis of biomass and organic acids. Thus, the increase in glycerol formation in strain TN4 was due to the large increase in formation of 2-oxoglutarate. More than 4 % of the carbon source in the anaerobic cultivations of the strain with cytoplasmic transhydrogenase was converted into 2-oxoglutarate, but the compound was also produced in slightly higher amounts in the cultivations of TN3 as compared to TN1. One explanation for this could be that presence of the control plasmid in multiple copies has a stress effect on the cell, which leads to leakage of the compound.

The intracellular concentrations of NAD(H) and NADP(H) were measured in cells of TN1, TN3 and TN4 sampled in the exponential growth phase. This was done to analyse the direction of the flux through the reaction catalysed by the soluble transhydrogenase in more detail. The direction of the reaction in equation 1 is determined by the size of Gibb's free energy of the reaction as described in equation 2.

$$\Delta G = -RT\ln((a_{NAD+,cyt} * a_{NADPH,\ cyt})/(a_{NADH,\ cyt} * a_{NADP+,cyt})) \qquad \text{(eq. 2)}$$

The standard Gibb's free energy, ΔG^0, for the reaction can be neglected. In order to calculate ΔG, the activities (or "free concentrations"), a_{NAD+}, a_{NADPH}, a_{NADH} and a_{NADP+}, of the four nucleotides have to be measured in the cytoplasm where the soluble transhydrogenase is active. Furthermore, isolation of the cytoplasm has to be carried out while maintaining anaerobic conditions during the proceedings. We found this beyond the scope of this paper and decided to measure the total level of the four nucleotides in the cells using a fast sampling technique, instead. Hence, in the further analysis it is assumed that the activities of the nucleotides are approximately equal to the concentrations and that the content of the four nucleotides in other compartments than the cytoplasm can be neglected. The latter assumption is supported by the fact that the number of mitochondria

in yeast is significantly reduced during anaerobic growth conditions as compared to aerobic conditions. No variations in the concentrations of the four nucleotides could be observed between samples from the early and the late exponential growth phase, respectively. In Table 3 it is seen that the ratio of NADPH/$NADP^+$ is approximately 35 times higher than the ratio of NADH/NAD^+ in strains TN1 and TN3.

TABLE 3

Strain	NAD^+	$NADP^+$	NADH	NADPH	NADH/ NAD^+	NADPH/ $NADP^+$
TN1	2.87 ± 0.09	0.23 ± 0.01	0.44 ± 0.01	1.21 ± 0.07	0.15 ± 0.01	5.26 ± 0.55
TN3	2.85 ± 0.11	0.24 ± 0.01	0.43 ± 0.01	1.19 ± 0.07	0.15 ± 0.01	4.96 ± 0.52
TN4	3.17 ± 0.07	0.27 ± 0.02	0.54 ± 0.02	0.80 ± 0.10	0.17 ± 0.01	2.96 ± 0.60

Table 3 *The intracellular concentrations of NAD(H) and NADP(H) in µmol per gram biomass (dry weight) in cells of strains TN1, TN3 and TN4 sampled during exponential growth in anaerobic glucose-limited batch cultivations.*

It can be calculated from the nucleotide concentrations in TN1 and TN3 that ΔG of a transhydrogenase reaction in these strains is 8.8-8.9 KJ/mole. This means that introduction of a reaction catalysed by transhydrogenase in these strains will result in conversion of NADPH and NAD^+ to NADH and $NADP^+$. Hence, the measurements of the intracellular concentrations of the four nucleotides support the findings from the measurements of the product formation in the three strains. Expression of transhydrogenase in TN4 results in some changes in the intracellular nucleotide levels. The NADPH/$NADP^+$ ratio is reduced to 2.96 while the NADH/NAD^+ ratio is almost constant, at 0.17. The reduction in the pool of NADPH strongly supports that transhydrogenase converts NADPH into NADH. The measurements also indicate that presence of transhydrogenase does not result in balancing of the redox level of the two-cofactor pools. This might be due to a very rigid regulation of the NADH/NAD^+ ratio as indicated by the constant value of this ratio in the three strains.

Concluding remarks

The direction of the reaction catalysed by transhydrogenase during other growth conditions. Measurements have not been published earlier of the intracellular concentrations of NAD(H) and NADP(H) in *S. cerevisiae* cells growing under anaerobic conditions. Thus, comparisons to the levels of these cofactors during other growth conditions must be made in order to evaluate the results obtained above. In *S. cerevisiae* cells grown in aerobic shake flasks the following values have been published (Sáez & Lagunas, 1976): 1.51 μmole NAD^+ per gram dry weight (1.5 ml cell volume per gram dry weight is assumed (de Koning & van Dam, 1992), 1.81 μmole NADH per gram dry weight, 0.21 μmole NADPH per gram dry weight and 0.04 μmole $NADP^+$ per gram dry weight. Some differences in the levels of the nucleotides are observed as compared to Table 3 but the ratios of NADPH/$NADP^+$ and NADH/NAD^+ of 5.25 and 1.20, respectively, indicate that also under aerobic conditions transhydrogenase would convert NADPH and NAD^+ to NADH and $NADP^+$. The high NADH/NAD^+ ratio could be due to a large flux through the tricarboxylic acid cycle during aerobic growth as compared to anaerobic growth. In a more recent study the intracellular concentrations of NADH and NAD^+ in *S. cerevisiae* cells that were preincubated anaerobically was determined to 0.37 μmole per gram dry weight and 1.63 μmole per gram dry weight, respectively (de Koning & van Dam, 1992). These data support the low NADH/NAD^+ found in this study. In *A. vinelandii* the nucleotide levels also indicate that the direction of the transhydrogenase reaction results in consumption of NADPH and formation of NADH. During aerobic conditions the ratios of NADH/NAD^+ and NADPH/$NADP^+$ was determined to 0.034 ± 0.018 and 0.251 ± 0.04 (Voordouw *et al.*, 1983). When the availability of oxygen was reduced to 20% of the maximum oxidation velocity of the cells, the two ratios changed to 0.214 and 0.316, respectively. Under these condition the reaction catalysed by transhydrogenase was close to equilibrium with ΔG equal to 1.0 KJ per mole.

By reducing the concentration ratio of NADPH/$NADP^+$ below that of NADH/NAD^+ in *S. cerevisiae* it is possible to change the direction of the transhydrogenase reaction towards consumption of NADH and formation of NADPH. This could be achieved by deletion of *MET19*, encoding glucose-6-phosphate dehydrogenase, which would result in elimina-

tion of the ability of the cell to reduce $NADP^+$ to NADPH through the pentose phosphate pathway.

The presence of transhydrogenation reactions in *S. cerevisiae*. The balance of the degree of reduction for the anaerobic cultivations of strains TN1 and TN3 closed within 2-3%. This means that the net formations of NADH and NADPH, respectively, have to balance as well. Through knowledge of the cofactor dependence of the reactions leading to synthesis of the products listed in Table 2, it was calculated that the net formation of NADH and NADPH was 19 mmoles per c-mole glucose and -17 mmoles per c-mole glucose, respectively. Thus, formation of glycerol can not account for reoxidation of the entire surplus pool of NADH formed in synthesis of biomass and organic acids. In order to balance the net formations of the two cofactors, coupled reactions in which NADH is converted into NADPH have to exist in *S. cerevisiae*. These could be almost identical reactions that only differ in their cofactor dependence. Such a role as a redox shunt between the two cofactor systems has been proposed in animal mitochondria for the reactions catalysed by two isoenzymes of isocitrate dehydrogenase (Sazanov & Jackson, 1994). The enzymes catalyse the reversible conversion of isocitrate into 2-oxoglutarate under simultaneous formation of NADH or NADPH, respectively. The proposed hypothesis suggests that a net conversion of surplus NADPH into NADH can be achieved by operating the NADH-dependent enzyme in the forward direction towards formation of 2-oxoglutarate while the NADPH-dependent enzyme operates in the reversed direction. Together with the membrane-bound transhydrogenase, present in animal cells, this system should have a role in the regulation of the tricarboxylic acid cycle activity. Genes encoding three isocitrate dehydrogenases have been identified in *S. cerevisiae* (Loftus *et al.*, 1994; Cupp & McAlister-Henn, 1991; Haselbeck & McAlister-Henn, 1991). The cytoplasmic enzyme, encoded by *IDP2*, is glucose-repressed and thus, not active during the growth conditions used in this work. The two mitochondrial enzymes, encoded by *IDH* and *IDP1*, are dependent on NADH and NADPH, respectively, and are active during growth on glucose. If the reactions encoded by isocitrate dehydrogenases function as a redox shunt in animal cells, this could also be the case in yeast. Hence, the system consisting of the coupled reactions catalysed by the two isocitrate dehydrogenases could have a role in conversion of surplus NADH, formed in synthesis of biomass and organic acids, into NADPH.

EXAMPLE 2

Expression of a transhydrogenase activity in *Lactococcus lactis* and *Escherichia coli*

Introduction

cth, encoding the cytoplasmic transhydrogenase from *Azotobacter vinelandii*, was cloned by PCR using primers BglII-cth (5'-tacgaagatctGCTGTATATAAC-TACGATGTGGTGG-3') (SEQ ID NO:9) and CTH-XhoI (5'-tagcactcgagt-taAAAAAGCCGATTGAGACC-3') (SEQ ID NO:10) and pfu polymerase. The resulting DNA fragment was digested with the restriction enzymes BglII and XhoI and inserted into the multi cloning site of the *E. coli/L. lactis* shuttle vector pTRKH2-p170 behind a strong constitutive derivate of the promoter p170 (S. M. Madsen, J. Arnau, A. Vrang, M. Givskov amd H. Israelsen (1999). *Molecular Microbiology* **32**, 75-87). The resulting plasmid was denoted pTRKH2-p170-cth. The promoter region of the vector and the inserted *cth* were sequenced, whereby it was verified that the gene had been inserted correctly into the shuttle vector.

E. coli DH5α and *L. lactis* subsp. *cremoris* were both transformed with pTRKH2-p170-cth and transformants were selected on plates containing complex medium (LB and GM13. respectively) supplemented with erythromycin. Independent pTRKH2-p170-cth transformants of both *E. coli* and *L. lactis* were grown in shake flasks in LB medium and GM13 medium, respectively, supplemented with erythromycin. In the late exponential growth phase, cell samples were withdrawn from the shake flasks and the protein pools of the cells were extracted.

The extracted protein pools were assayed for activity of the cytoplasmic transhydrogenase. The results are listed below

Microorganism	Transhydrogenase activity (U per mg protein)
E. coli DH5α	not detectable
E. coli DH5α pTRKH2-p170-cth	0.568
L. lactis subsp. *cremoris*	not detectable
L. lactis subsp. *cremoris* pTRKH2-p170-cth	0.107

From the data it was concluded that the cytoplasmic transhydrogenase had been successfully expressed in both *E. coli* and *L. lactis*.

5

REFERENCES

Altschul, S.F., Gish, W., Webb, M., Myers, E.W. & Lipman,D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215** p. 403-410.

Ansell R, Granath K, Hohmann S, Thevelein JM & Adler L (1997). The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. *EMBO J.* **16** p. 2179-2187.

Bergmeyer, H. U. (1985). Methods in enzymatic analysis. Vol. VI, VCH publishers, Deerfield Beach, FL.

Bjellqvist, B.,Hughes, G.J., Pasquali, Ch., Paquet, N., Ravier, F., Sanchez, J.-Ch., Frutiger, S. & Hochstrasser, D.F. (1993). The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **14** p. 1023-1031.

Bruinenberg, P.M., de Bot, P.H.M. & Scheffers, W.A. (1983). An enzymic analysis of NADPH production and consumption in *Candida Utilis*. *J. Gen. Microbiol.* **129** p. 965-971.

Bruinenberg, P.M., van Dijken, J.P. & Scheffers, W.A. (1982). A theoretical analysis of NADPH production and consumption in yeasts. *J.Gen.Microbiol.* **129** p. 953-964.

Cohen P.T.& Kaplan N.O. (1970). Purification and properties of the pyridine nucleotide transhydrogenase from Pseudomonas aeruginosa. *J Biol Chem* **245** p. 2825-2836

Cupp, J. R. & McAlister-Henn, L. (1991). NAD^+-dependent isocitrate dehydrogenase. Cloning, nucleotide sequence, and disruption of the *IDH2* gene from *Saccharomyces cerevisiae*. *J. Biol.Chem.* **266** p. 22199-22205.

de Koning, W. & van Dam, K. (1992). A method for determination of changes of glycolytic metabolites in yeast on a subsecond time scale using ectraction at neutral pH. *Anal.Biochem.* **204** p. 118-123.

Eriksson, P., Andre, L., Ansell, R. Blomberg, A. & Adler, L. (1995). Cloning and characterization of GPD2, a second gene encoding sn- glycerol 3-phosphate dehydrogenase (NAD+) in *Saccharomyces cerevisiae*, and its comparison with GPD1. *Mol Microbiol* **17** p. 95-107

French, C.E., Boonstra, B., Bufton, K.A.J. & Bruce, N.C. (1997). Cloning , sequence, and properties of the soluble nucleotide transhydrogenase of *Pseudomonas fluorescens*. *J. Bact.* **179** p. 2761-2765

Haselbeck, R. J. & McAlister-Henn, L. (1991). Isolation, nucleotide sequence, and disruption of the *Saccharomyces cerevisiae* gene encoding mitochondrial NADP(H)-specific isocitrate dehydrogenase. *J.Biol.Chem.* **266** p. 2339-2345.

Hojeberg, B., Brodelius, P., Rydstrom, J. & Mosbach K. (1976). Affinity chromatography and binding studies on immobilized 5'-monophosphate and adenosine 2',5'-bisphosphate of nicotinamide nucleotide transhydrogenase from Pseudomonas aeruginosa. *Eur J Biochem* **15** p. 467-475.

Jones, E.W. & Fink, G.R. (1982). Regulation of amino acid and nucleotide biosynthesis in yeast. In *The molecular biology of the yeast Saccharomyces. Metabolism and gene expression*. P. 181-299. Edited by J.N. Strathern, E.W. Jones & J.R. Broach. Cold Spring Harbor Laboratory.

Kuriyan, J., Krishna, T.S.R., Wong, L., Guenther, B., Pahler, A., Williams Jr, C.H. & Model, P. (1991). Convergent evolution of similar function in two structurally divergent enzymes. *Nature* **352** p. 172-174.

Larsson K, Ansell R, Eriksson P & Adler L (1993). A gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) complements an osmosensitive mutant of Saccharomyces cerevisiae. *Mol Microbiol* **10** p. 1101-1111.

Loftus, T. M, Hall, L. V., Anderson, S. L. & McAlister-Henn, L. (1994). Isolation, characterization, and disruption of the yeast gene encoding cytosolic NADP-specific isocitrate dehydrogenase. *Biochemistry* **33**, p. 9661-9667.

Meaden, P.G., Dickinson, F.M., Mifsud, A., Tessier, W., Westwater, J., Bussey, H. & Midgley, M.(1997). The ALD6 gene of Saccharomyces cerevisiae encodes a cytosolic, Mg(2+)- activated acetaldehyde dehydrogenase. *Yeast* **13** p. 1319-1327.

Middleditch, L.E., Atchison, R.W. & Chung, A.E.. (1972). Pyridine nucleotide transhydrogenase from Azotobacter vinelandii. Some aspects of its structure. *J Biol Chem* **10** p. 6802-6809 *Mol Microbiol* **10** p. 1101-1111.

Moye, W. S., Amuro, N., Rao, J. K. & Zalkin, H. (1985). Nucleotide sequence of yeast GDH1 encoding nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase. *J Biol Chem* **260** p.8502-8508.

Nissen, T.L., Schulze U., Nielsen, J. & Villadsen, J. (1997). Flux distributions in anaerobic, glucose-limited cultures of *Saccharomyces cerevisiae*. *Microbiology* **143**, p. 203-218.

Olausson, T., Fjellstrom, O., Meuller, J. & Rydstrom, J. (1995). Molecular biology of nicotinamide nucleotide transhydrogenase--a unique proton pump. *Biochim Biophys Acta* **15** p. 1-19.

Oura, E. (1977). Reaction Products of Yeast Fermentations. *Proc. Biochem.* **12**, p. 19-21+35

Postma, E., Verduyn, C., Scheffers, W. A. & van Dijken, J. P. (1989). Enzyme analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **53**, 468-477.

Rydström, J. & Hoek, J.B. (1988). Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem. J.* **254** p. 1-10.

Rydström, J., Hoek, J.B. & Ernster, L. (1976) in *The enzymes* (Boyer, P.D., ed.) 3rd edn. Vol. XIII, p. 51-88, Academic Press, New York, London.

Rydström, J., Persson, B. & Carlenor, E. (1987). Transhydrogenases linked to pyridine nucleotides. *In Pyridine nucleotide coenzymes: Chemical, biochemical, and medical aspects*. **2B** p. 433-460. Edited by D. Dolphin, R. Poulson & O. Avramovic. John Wiley & Sons, Inc.

Sáez, M.J. & Lagunas, R. (1976). Determination of intermediary metabolites in yeast. Critical determination of the effect of sampling conditions and recommendations for obtaining true levels. *Mol.Cell.Biochem.* **13** p. 73-78.

Sambrook, J., Fritsch. E.F. & Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, N.Y.

Sazanov, L.A. & Jackson, J.B. (1994). Proton-translocating transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenases operate in a substrate cycle which contributes to fine regulation of the tricarboxylic acid cycle activity in mitochondria. *FEBS letters*. **344** p. 109-116.

Schiestl RH & Gietz RD (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16** p. 339-346

Tao, K., Makino, K., Yonei, S., Nakata, A. & Shinagawa, H. (1989). Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: Homologies between OxyR protein and a family of bacterial activator proteins. *Mol. Gen. Genet.* **218** p. 371-376.

Triglia, T., Peterson, M.G. & Kemp, D.J. (1988). A procedure for *in vitro* amplification of DNA that lie outside the boundaries of known sequences. *Nuc.Acid.Res.* **16** p. ???

van Dijken, J.P. & Scheffers, W.A. (1986). Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiology Reviews* **32** p. 199-224.

Verduyn, C., Postma, E., Scheffers, W.A. & van Dijken, J.P. (1990). Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J.Gen.Microbiol.* **136** p. 395-403.

Voordouw G., van der Vies S.M., Eweg J.K., Veeger C., van Breemen J.F., van Bruggen E.F. (1980). Pyridine nucleotide transhydrogenase from Azotobacter vinelandii. Improved purification, physical properties and subunit arrangement in purified polymers. *Eur J Biochem.* **111** p. 347-355.

Voordouw G., Veeger C., Van Breemen J.F. & Van Bruggen E.F. (1979). Structure of pyridine nucleotide transhydrogenase from Azotobacter vinelandii. *Eur J Biochem.* **98** p. 447-454.

Voordouw, G., van der Vies, S. M. & Themmen, A. P. N. (1983). Why are two different types of transhydrogenases found in living organisms? *Eur.J.Biochem.* **131** p. 527-533

Wach, A., Brachat, A., Pöhlmann, R. & Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruption in *Saccharomyces cerevisiae*. *Yeast* **10**. 1793-1808.

Walfridsson, M., Anderlund, M., Bao, X. & Hahn-Hägerdahl, B. (1997). Expression of different levels of enzymes from the *Pichia stipidis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effect on product formation during xylose utilisation. *Appl. Microbiol. Biotechnol.*

Wenzel, T.J., van den Berg, M.A., Visser, W., van den Berg, J. & Steensma, H.Y. (1992). Characterization of *Saccharomyces cerevisiae* mutants lacking the E1α subunit of the pyruvate dehydrogenase complex. *Eur.J.Biochem.* **209**. P. 697-705.

Widmer, F. & Kaplan, N.O. (1977). Pseudomonas aeruginosa transhydrogenase: affinity of substrates for the regulatory site and possible hysteretic behavior. *Biochem Biophys Res Commun* **20** p. 1287-1292.

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1-3-3	Date of deposit	26 June 1998 (26.06.1998)
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5-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
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5-3-3	Date of deposit	26 June 1998 (26.06.1998)
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